Lipid Peroxide Levels and Superoxide-Scavenging Abilities of Sera Obtained from Hotbred (Thoroughbred) Horses

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ABSTRACT. Hotbred (Thoroughbred) horses were grouped into three classes according to the levels of constant physical exercise (foals, 6 months old; racing horses, 5 years old; horses for breeding, 6–10 years old), and lipid peroxide levels in their sera were measured as thioburbituric acid-reactive substances. No significant differences were observed among them. The superoxide-scavenging abilities of sera were measured next; to examine the antioxidative properties of hotbreds, and were found to be highest in the racing horses. The higher scavenging ability of the racing horses might contribute to keep their lipid peroxide levels as low as those of the other two groups. HPLC analysis of substances in sera suggested that the presence of albumin-bound bilirubin was one of the reasons for the high superoxide-scavenging ability of sera of the racing horses. When the hotbreds were compared with coldbred (crossbred) horses, the lipid peroxide level of hotbreds was higher (7.0 ± 1.2 nmol/m*l*) than that of coldbreds (2.6 ± 0.7 nmol/m*l*). Comparison of the superoxide-scavenging abilities of sera between hotbreds and coldbreds showed that the hotbreds possessed higher scavenging ability than the coldbreds. These results indicated that the lipid peroxide level in sera of hotbreds was higher than that of coldbreds regardless of the higher superoxide-scavenging abilities of sera.—KEY WORDS: bilirubin, hotbred, lipid peroxide, serum, superoxide.

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Evidence to support a relationship between severe physical exercise, oxygen consumption and oxygen radical damage has been provided by the observation of tissue damage, and measurements of products of lipid peroxidation in men and animals [3, 4, 7, 8]. Hotbred (Thoroughbred) horses are thought to consume much more oxygen than coldbred horses because of their constant physical exercise. The present study was carried out to evaluate the effects of constant physical exercise on hotbreds. For this purpose, hotbreds were divided into three groups (foals, racing horses and horses for breeding), and the lipid peroxide levels in sera obtained from them, as well as coldbreds, were measured. Furthermore, using the spin-trapping method, the relationship between the lipid peroxide levels and the antioxidizing levels in sera was analyzed by measuring the superoxide-scavenging abilities of sera obtained from the three hotbred groups and one coldbred group. A method combining spin trapping and high-performance liquid chromatography was also employed to identify the substances having superoxide-scavenging ability in sera.

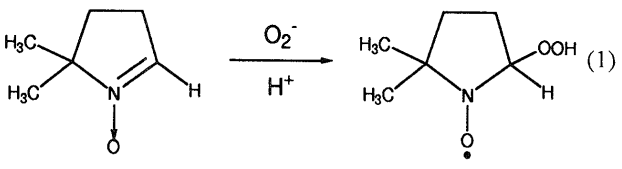
MATERIALS AND METHODS

Chemicals: Sodium thiobarbituric acid (TBA), 1,1,3,3-tetraethoxy propane (TEP), butylated hydroxytoluene (BHT), and other chemicals were purchased from Wako Pure Chemical Industries Ltd. Hypoxanthine (HXn), xanthine oxidase (XOD) and 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) were obtained from Sigma Chemical Co. Bovine milk whey containing immunoglobulin G (IgG), bovine serum albumin (BSA) and β-lactgloblin (β-Lg) was kindly provided by Dr. K. Shimazaki, Faculty of Agriculture, Hokkaido University.

Sera: Sera were obtained from hotbreds (Thoroughbreds) and coldbreds (crossbred between Percheron and Breton). The sera from the Thoroughbreds was divided into three groups (foals, 6 months old, 4 colts and 5 fillies; racing horses, 5 years old, 37 males and 8 females; horses for breeding, 6–10 years old, 3 males, 33 females).

Lipid peroxide levels in sera: The lipid peroxide levels in sera were determined by the TBA-reaction method [1]. TEP, which quantitatively converts to malondialdehyde during the reaction, was used as a standard. The lipid peroxide levels were expressed as nmol of thioburbuturic-reactive substances (TBARS) in 1 m*l* of serum.

Superoxide-scavenging abilities of sera: The oxidation reaction of HXn by XOD was employed to obtain superoxides (O_2^-) [10]. Superoxides are short-lived and, therefore, unobservable by usual electron-spin resonance (ESR) techniques, but ESR spectrometry combined with the spin-trapping technique is now available for quantitative observation of superoxides [6]. When DMPO is used as a spin-trapping reagent, superoxides are transformed to nitroxide free radicals according to the following reaction. The resulting nitroxide free radicals (DMPO-OOH adducts) are relatively long-lived and become ESR observable, and the ESR signal intensities give quantitative measurements



(1)

of superoxides. If substance (R) having a superoxide-scavenging ability coexists with DMPO in the reaction solution, the following reaction also occurs in addition to reaction (1).

$$R + O_2^- \rightarrow R^- + O_2 \text{ [or } RO_2^-\text{]} \quad (2)$$

Reaction (2) competes with reaction (1) and, therefore, the ESR signal intensity of DMPO-OOH becomes small. The superoxide-scavenging ability of serum was measured according to this idea. The experimental procedures were as follows. We used doubly-distilled water in which the tiny amounts of transition metals retained were purified by a column with a chelating resin (Chelex® 100 Resin, Bio Rad Lab.). This water was used for the preparation of all the reaction solutions. One gram of DMPO was dissolved in 1 m*l* of the water. Impurities in DMPO were removed by adding one gram of activated charcoal (Norit® A, Nacalai Tesque) into the solution, stirring for 20 min and filtering with filter paper (Advantic Toyo). This provided aqueous solutions containing 0.4–0.6 M DMPO. A mixed solution of 80 μl of 0.1 M phosphate buffer (pH 7.4), 50 μl of 1 mM HXn, 50 μl of DMPO solution and 10 μl of serum (or 10 μl of 0.1 M phosphate buffer [pH 7.4] for control) was then prepared. As soon as 10 μl of XOD (0.114 U/m*l*) was added to the solution, it was transferred to a quartz flat [4 × 1 × 0.02 cm^3) cell for ESR measurements. ESR measurements were performed on a JEOL JES RE1X spectrometer at room temperature. The magnetic field was 334.5 ± 10 mT, the field modulation was 100 kHz with an amplitude of 0.1 mT, and the incident microwave power level was maintained at 10 mW.

ESR signal intensity increases with time after addition of XOD, reaches a maximum at 5 min and is maintained at a plateau for 7 min. The superoxide-scavenging ability of serum was calculated as follows.

$$O_2^-\text{-scavenging ability} = \frac{[\text{ESR intensity}]_O - [\text{ESR intensity}]_R}{[\text{ESR intensity}]_O} \times 100\ (\%) \quad (3)$$

where $[\text{ESR intensity}]_O$ and $[\text{ESR intensity}]_R$ represent the maximum ESR signal intensities in the absence and presence of serum, respectively.

High-performance liquid chromatography (HPLC) of sera and superoxide-scavenging ability of each fraction: Substances having superoxide-scavenging ability in sera were separated using an HPLC system (TOSOH CCP & 8020] equipped with a TSKgel G3000 SWXL column (7.8 mm inner diameter, 30 cm long) and a TSKgel G2000 SWXL column (7.8 mm inner diameter 30 cm long) by turns. The volume of serum injected was 500 μl. The elution solution consisted of 25 mM phosphate buffer and 0.3 M NaCl (pH 6.7). The flow rate was 0.7 m*l*/min and the eluent was monitored by UV detector (TOSOH UV-8020) at 280 nm. The superoxide-scavenging ability of each fraction was measured as above. Bovine milk whey containing IgG, BSA and β-Lg was used as a standard for estimating molecular weights and to check the resolution of HPLC.

Measurements of proteins: The protein content of each fraction obtained by HPLC of sera was measured by the method of Lowry *et al.* [11].

Measurements of bilirubin: The presence of bilirubin in HPLC fractions was confirmed by both optical absorbance spectrometry λ_{max} = 460 nm) and van den Bergh reaction using diazobenzenesulfonic acid [12].

Statistical analysis: Statistical analyses were carried out using Student's *t*-test. A probability level of less than 5% was regarded as significant.

RESULTS

Figure 1 shows the lipid peroxide levels (TBARS) of sera obtained from coldbreds and hotbreds (horses for breeding, racing horses and foals). No statistically significant difference in the TBARS was observed among the three groups of hotbreds, but a significant difference between hotbreds and coldbreds was observed at**p<0.01. This meant that hotbreds possessed a naturally higher lipid peroxide level than coldbreds regardless of the presence or absence of constant exercise. It is noted that similar value of TBARS were observed regardless of the differences between the sexes (data not shown).

The spin-trapping method with DMPO revealed the superoxide-scavenging abilities of sera obtained from coldbred and hotbred horses as shown in Fig. 2. The results showed that (1) the highest superoxide-scavenging ability was observed in the sera of racing horses, with a statistical level of significance of *p<0.05, regardless of the lack of differences in the lipid peroxide levels, and (2) the scavenging abilities of sera obtained from hotbreds were higher than those of coldbreds, (statistical level of significance of *p<0.05 between horses for breeding and coldbreds, as well as between foals and coldbreds, and of

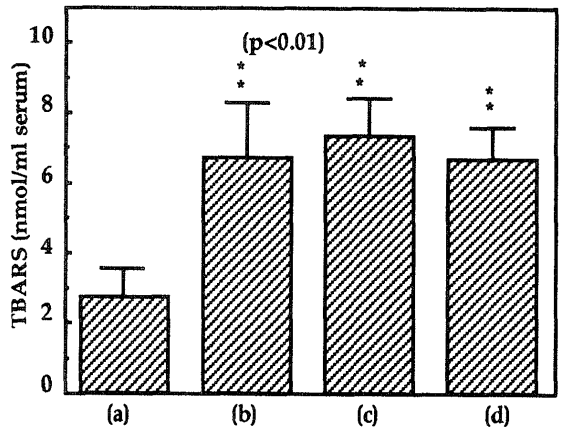


Fig. 1. TBARS (nmol/m*l* serum) in sera obtained from (a) coldbreds, (b) hotbreds for breeding, (c) racing hotbreds and (d) hotbred foals. The difference in TBARS between coldbreds and hotbreds was statistically significant (**p<0.01).

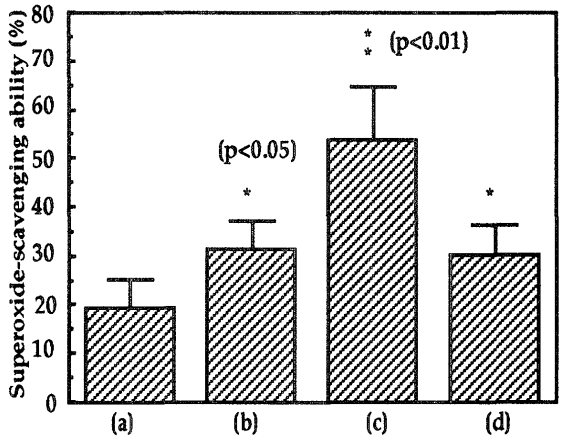


Fig. 2. Superoxide-scavenging abilities of sera obtained from (a) coldbreds, (b) hotbreds for breeding, (c) racing hotbreds and (d) hotbred foals. The statistical significance of the difference between coldbreds and horses for breeding, and foals, was *0.01<p<0.05, that between coldbreds and racing horses **p<0.01, and that between racing horses and horses for breeding, and foals, was *0.01<p<0.05.

**p<0.01 between racing horses and coldbreds).

The substances in sera having superoxide-scavenging ability were next analyzed by a method combining HPLC and spin trapping. The substances in sera were first separated by HPLC with two gel-filtration columns (TSKgel G2000SWXL and G3000SWXL) and then the superoxide-scavenging ability of each fraction was measured by the spin-trapping method with DMPO. Figure 3a shows an elution profile of serum obtained from a horse for breeding. X- and Y-axes represent the elution volume of HPLC and superoxide-scavenging ability, respectively. Figures 3b and 3c show elution profiles of sera obtained from a racing horse and a coldbred, respectively. The positions corresponding to IgG, BSA and β-Lg are illustrated by arrows.

Overall inspection of the three profiles indicated that the superoxide-scavenging abilities of sera from the horses for breeding and the racing horses were higher than the superoxide-scavenging abilities of the sera from coldbreds. The profiles denoted by open circles show the results obtained in the presence of KCN. Mammals possess enzymes or proteins with superoxide-scavenging ability in sera, several of which are known to be inhibited by cyanide (CN^-) [15]. Cu-, Zn-superoxide dismutase (Cu, Zn-SOD), extracellular SOD (EC-SOD) and ceruloplasmin are inhibited by cyanide, whereas Mn-SOD and albumin-bound bilirubin (Alb-BR) are not. In Fig. 3a, it can be seen that superoxide-scavenging substances were found in fractions corresponding to elution volumes 15–16 and 17.5 m*l*. The fractions eluted at 15–16 m*l* were cyanide-sensitive. These fractions eluted near the molecular weight of 134 K, and cyanide-sensitive ceruloplasmin (mw 134 K) was assigned to these fractions. This assignment was further confirmed by the ferrooxidase activity of these fractions [14]. Another fraction at 17.5 m*l* was not inhibited by cyanide. Mn-SOD

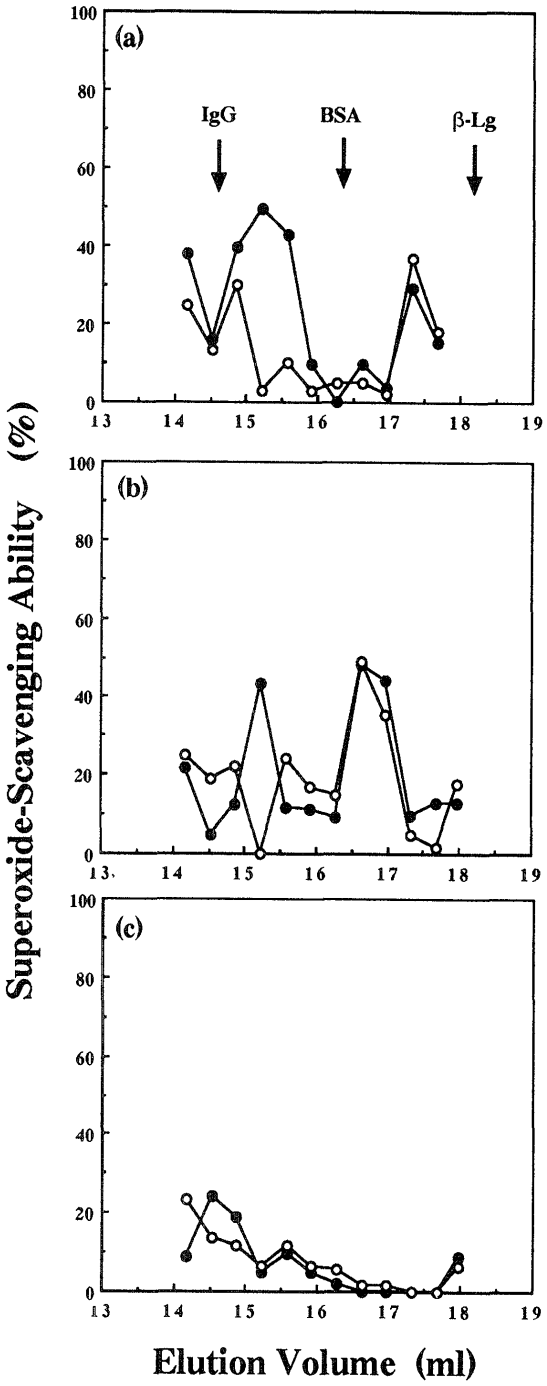


Fig. 3. HPLC profiles of sera obtained from (a) a horse for breeding, (b) a racing horse and (c) a coldbred judged by superoxide-scavenging ability. X-axis stands for the elution volume (m*l*). Arrows indicate the positions where IgG, BSA and β-Lg elute. —●—, superoxide-scavenging ability measured in the absence of KCN; —○—, superoxide-scavenging ability measured in the presence of KCN.

(mw 40 K) and Alb-BR (67 K) did not correspond to these fractions, as judged by their molecular weights, and the exact identities of these fractions have not been determined. It is noted that low superoxide-scavenging activity was

observed in fractions at elution volumes 16.5–17 m*l* in which high activity due to Alb-BR was observed in racing horses (see below).

Figure 3b shows the elution profile of serum obtained from a racing horse. A superoxide-scavenging substance was found in fraction at 15.5 m*l*. This fraction was thought to correspond to those at 15–16 m*l* obtained from the horse for breeding. For the reasons reported above, the fraction at 15.5 m*l* was regarded as ceruloplasmin. The fractions at 16.5–17 m*l* were newly observed, and these fractions had a large ability to scavenge superoxides. They eluted near the molecular weight of BSA, and were cyanide-insensitive. Examination by both optical absorbance spectrometry and van den Bergh reaction using diazobenzenesulfonic acid proved that bilirubin was present (2.3 mg/d*l*), and the substance in these fractions was, therefore, identified as Alb-BR.

The HPLC-elution profile of serum from coldbreds is shown in Fig. 3c. The overall superoxide-scavenging ability was low compared with the abilities of the hotbreds, and it was concluded that Alb-BR was almost absent since the amount of bilirubin in the fractions at 16.5–17 m*l* was quite low (0.4 mg/d*l*). The presence of ceruloplasmin was also not clear.

The measurement of the protein content in each fraction by Lowry's method [8] gave an elution profile consisting mainly of two peaks at 14–15 and 16–17 m*l*, corresponding to IgG and BSA, respectively. Quite similar profiles were obtained from all sera examined (data not shown), and the results indicated that there were no differences in serum protein contents between horses for breeding and racing horses, or between hotbreds and coldbreds.

DISCUSSION

In the present study no effects of constant exercise on racing horses were observed because of the originally high level of lipid peroxidation in sera of hotbreds (Fig. 1). The sera of foals already had high lipid peroxide levels regardless of the absence of constant exercise, whilst the racing horses had a greater superoxide-scavenging ability than horses for breeding or foals (Fig. 2). This means that the racing horses can withstand the excessive uptake of oxygen due to constant physical exercise, that is, the high superoxide-scavenging ability of sera of racing horses may contribute to keeping their lipid peroxide levels as low as those of foals and horses for breeding. HPLC analysis proved that the high activity of Alb-BR to scavenge superoxides was present in racing horse sera. Bilirubin is produced from ferroprotoporphyrin through several enzymatic reactions [13], therefore, it seems necessary to examine the elevation of the activities of these enzymes in racing horses in another study.

Lipid peroxidation is caused by activation of either lipids or molecular oxygens. Enzymatic oxidation of lipids by lipoxygenase and cycloxygenase [19], and autoxidation of lipids by metal ions [20], start from the activation of lipids, whereas molecular oxygens are activated by microsome, peroxisome, and flavoproteins [17], and by toxic drugs through redox cycle [9]. Lipid peroxides are decomposed by glutathione peroxidase (GSH·Px) [2], glutathione *S*-transferase (GSH·Tr) [16], Fe^{3+} (or Cu^{2+})-ascorbic acid system [5], etc. In some cases, before the formation of lipid peroxides, reactive oxygens are inactivated by several enzymes and proteins (SODs, catalase, GSH·Px, ceruloplasmin, Alb-BR, metallothionein, transferrin, lactoferrin and ferritin) and α-tocopherol, ascorbic acid and uric acid. These substances are present not only in serum but also in tissues and organs such as the liver, lung, brain, kidney, etc., and the defense system against oxidative stress must be evaluated by all antioxidative activities in the body. The balance between the generation and decomposition of lipid peroxides may determine their concentration in serum. In the present study, the substances with superoxide-scavenging activity in serum were examined as a part of the total antioxidative activities which horses possessed. In racing horses constant physical exercise was responsible for the oxidative stress. The increase in the amount of Alb-BR may be one quick response to achieve the defence system. Alb-BR scavenges reactive oxygens, and the reactions of bilirubin with reactive oxygens produce several oxidative substances which can be excreted into urine [18]. In racing horses it is inferred that the excretion of bilirubin is accelerated by reactions with reactive oxygens and that its accumulation in serum is thereby suppressed.

Comparison of the hotbreds and coldbreds showed that the superoxide-scavenging abilities of sera of hotbreds were higher than those of coldbreds. This was an unexpected result because it is generally accepted that the higher the superoxide-scavenging ability of serum, the lower the lipid peroxide level in serum. Nevertheless, the hotbreds had higher lipid peroxide levels than coldbreds. Hotbreds may genetically possess high lipid peroxide levels, however, the factors which determine the high lipid peroxide level in sera of hotbred horses have not been elucidated yet.

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REFERENCES

1. Buege, J. A. and Aust, S. D. 1987. Microsomal lipid peroxidation. *Methods Enzymol.* 52: 302–310.
2. Christphersen, B. O. 1969. Reduction of linolenic acid hydroperoxide by a glutathione peroxidase. *Biochim. Biophys. Acta* 176: 463–470.
3. Davies, K. J. A., Quintanilha, A. T., Brooks, G. A., and Packer, L. 1982. Free radicals and tissue damage produced by exercise. *Biochem. Biophys. Res. Commun.* 107: 1198–1205.
4. Dillard, C. J., Litov, R. E., Savin, W. M., Dumelin, E. E., and Tappel, A. L. 1978. Effects of exercise, vitamin E, and ozone on pulmonary function and lipid peroxidation. *J. Appl. Physiol.* 45: 927–932.
5. Donovan, D. H. and Menzel, D. B. 1978. Mechanisms of

lipid peroxidation: Iron catalyzed decomposition of fatty acid hydroperoxides as the basis of hydrocarbon evolution *in vivo*. *Experientia* 34: 775–776.
6. Finkelstein, E., Rosen, G. M., and Raukman, E. J. 1980. Spin trapping of superoxide and hydroxyl radical: Practical aspects. *Archiv. Biochem. Biophys.* 200: 1–16.
7. Gutteridge, J. M. C., Westermarck, T., and Halliwell, B. 1985. pp. 99–139. Oxygen Radical Damage in Biological Systems.
8. Ichikawa, I. 1987. Exercise and stress, pp. 123–144. *In*: Lipid Peroxidation in Biological Systems (Uchiyama, M., Matsuo, M., and Sagai, M. eds.), Gakkaishuppan Center, Tokyo (in Japanese).
9. Kappus, H. and Sies, H. 1981. Toxic drug effects associated with oxygen metabolism: Redox cycling and lipid peroxidation. *Experientia* 37: 1233–1241.
10. Krenitsky, T. A., Tuttle, J. V., Cattau, E. L., and Wang, P. 1974. A comparison of the distribution and electron acceptor specificities of xanthine oxidase and aldehyde oxidase. *Comp. Biochem. Physiol.* 49B: 687–703.
11. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193: 265–275.
12. Michaëlsson, M. 1961. Bilirubin determination in serum and urine (studies on diazo methods and a new copper-azo pigment method). *Scand. J. Clin. Lab. Invest.* 13 (*Suppl.* 56): 1–80.
13. Onishi, S., Isobe, S., Itoh, S., Ogino, T., Yokoyama, T., and Yamakawa, T. 1984. Structure and thermal interconversion of cyclobilirubin IXα. *Biochem. J.* 218: 667–676.
14. Osaki, S., Johnson, D. A., and Frieden, E. 1966. The possible significance of the ferrous oxidase activity of ceruloplasmin in normal human serum. *J. Biol. Chem.* 241: 2746–2751.
15. Oyanagi, Y. 1989. SOD and active oxygen modulators, p. 62. Nihon Igakukan, Tokyo (in Japanese).
16. Prohaska, J. R. 1980. The glutathione peroxidase activity of glutathione *S* transferase. *Biochim. Biophys. Acta* 611: 87–98.
17. Puglia C. D. and Powell, S. R. 1984. Inhibition of cellular antioxidants: A possible mechanism of toxic cell injury. *Environm. Health Perspectives* 57: 307–311.
18. Yamaguchi, T., Komuro, A., Nakano, Y., Tomita, M., and Nakajima, H. 1993. Complete amino acid sequences of bilirubin-IXβ. *Biochem. Biophys. Res. Commun.* 197: 1518–1523.
19. Vliegenthart, J. F. G. and Veldink, G. A. 1982. Lipoxygenase. pp. 29–64. *In*: Free Radicals in Biology (Pryor, W. A. ed.), Academic Press, New York.
20. Weiss, J. J. 1953. The auto-oxidation of ferrous ions in aqueous solution. *Experientia* 9: 61–62.