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OXIDATION OF TRIS TO ONE-CARBON COMPOUNDS IN A RADICAL-PRODUCING MODEL SYSTEM, IN MICROSOMES, IN HEPATOCYTES AND IN RATS

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The buffer substance tris(hydroxymethyl)aminomethane (Tris) is converted to formaldehyde in an hydroxyl radical producing model system and in rat liver microsomes, and to CO_2 in rat hepatocytes and in the intact rat. In microsomes, formaldehyde formation from Tris is inhibited by catalase, by the antioxidant propylgallate and by the iron chelator deferoxamine. formaldehyde formation is stimulated by the addition of Fe (II) EDTA. In hepatocytes, the formation of [^{14}C] CO_2 from [^{14}C] Tris is inhibited by propylgallate and by the iron chelator o-phenanthroline and is stimulated by the presence of a xanthine oxidase system plus Fe (II) EDTA in the medium. In the intact rat, the administration of [^{14}C] Tris results in the exhalation of [^{14}C] CO_2. The results indicate that an oxidant formed via a Fenton-type reaction, possibly the hydroxyl radical, may be involved in the formation of one-carbon compounds from Tris.

KEY WORDS: Hydroxyl radicals, tris(hydroxymethyl)aminomethane, hepatocytes, formaldehyde production, CO_2 production, propyl gallate, ortho-phenanthroline.

INTRODUCTION

Tris has been used for the correction of acidosis in clinical practice since its pharmacological properties were first described by Nahas in 1961.[1] The half-life of Tris in healthy volunteers is about 6 h; 82% of the unchanged drug is recovered in the urine within 24 h[2]. Apparently, thus far no metabolites have been observed. Christensen and Clifford[3] reported that α-hydroxymethylserine, a likely product of metabolic oxidation, was not formed from Tris *in vivo*, and Holmdahl and Nahas[4] did not observe exhalation of CO_2. Experimental experience shows that in the determination of microsomal demethylation reactions via measurement of formaldehyde with the Nash method[5] high blanks are found when Tris buffer is used. Since this may be due to the formation of formaldehyde from Tris, we investigated whether degradation of Tris to one-carbon compounds occurs and which mechanisms are involved. The results show that Tris is converted to CO_2 via formaldehyde by an oxidant formed via a Fenton-type reaction. This agent may be OH· or a species possessing oxidant properties similar to OH·.

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METHODS

Aqueous Model System

Unlabeled Tris was obtained from Merck, Darmstadt, FRG. Formaldehyde formation form Tris was studied in an aqueous OH· producing model system (Udenfriend system) consisting of 1.5 mM ascorbic acid, 50 μM $FeCl_3$ and 100 μM ethylenediaminetetraacetic acid, tetrasodium salt (EDTA) in 50 mM potassium phosphate buffer pH 7.4. Incubation was performed for 120 min at 37°C. Formaldehyde was quantified according to Nash.[5] The identity of the reaction product was verified by the formaldehyde dehydrogenase reaction.[6]

Microsomes

Liver microsomes were prepared from phenobarbital-pretreated (3 × 80 mg/kg in 24 h intervals, last injection 24 h prior to sacrifice) male Wistar rats (ca. 200 g) by differential centrifugation. Formation of formaldehyde was studied in 1 ml incubations with 1.5 mg protein/ml, 0.5 mM NADPH, a NADPH regenerating system consisting of 8 mM sodium isocitrate, 20 μl/ml isocitrate dehydrogenase and 3 mM $MgCl_2$, and 0.5 mM sodium azide in 66 mM potassium phosphate buffer pH 7.4. Formaldehyde was assayed according to Nash.[5] The identity of the reaction product was verified by the formaldehyde dehydrogenase reaction.[6] OH· or a species possessing oxidant properties similar to OH· was determined via formation of ethylene from methional as previously described.[7] Antioxidants were added in 10 μl acetone; the same volume of acetone was added to control incubations.

Hepatocytes

Freshly isolated hepatocytes were obtained from untreated male Wistar rats (250–300 g) according to a modification of the procedure described by Seglen.[8] Viability was determined by Trypan blue exclusion. Incubation of 3 × 10^6 cells with a tracer dose of 2.5 μ Ci (92.5 kBq) [^{14}C] Tris (0.15 mM) labeled at the hydroxymethyl groups - obtained by customer synthesis from Amersham Buchler, GmbH, Braunschweig, FRG, at a specific activity of 5.5 mCi (203.5 MBq)/mmol - was performed in 3 ml Ca^{2+}-containing Hepes buffer pH 7.4 under a continuous stream of air at 37°C for 60–120 min. Trapping of CO_2 was performed by a gas wash bottle arrangement described by Lauterburg and Bircher.[9] Blanks obtained for CO_2 formation from Tris in the absence of hepatocytes were subtracted. In some experiments, 0.25 μCi (9.25 kBq) [^{14}C] formaldehyde (8.3 μM) was used instead of Tris.

ANIMALS

Male Wistar rats (body weight ca. 250 g) were used. Cannula implantation was performed under pentobarbital anesthesia (50 mg/kg). A polyethylene silastic tubing of 0.5 mm inner diameter and 1 mm outer diameter was inserted into the jugular vein, the tip being placed into the right atrium. The cannula was fixed in the neck of the animal. The rats were allowed to recover for 24 h and were then brought into a restraining cage connected to a gas wash bottle arrangement described by Lauterburg and Bircher.[9] The animals received [^{14}C] Tris either as an intravenous bolus of 2–5 μCi

(74–185 kBq), using a solution of 10 μCi (370 kBq)/ml 2 M Tris, or as a continuous intravenous infusion with 2.5 μCi (92.5 kBq)/h (1.9 ml/h) between 0 and 15 min and subsequently with 0.5 μCi (18.5 kBq)/h (0.38 ml/h) between 15 and 140 min, using a solution of 1 μCi (37 kBq)/ml 0.2 M Tris. Total output of [^{14}C] CO_2 was measured by continuous collection of the expired CO_2. For this, air was drawn through the cage and the gas wash bottles at 0.5 ml/min. CO_2 was trapped in hyamine and ^{14}C was quantified by liquid scintillation counting.

RESULTS

Experiments carried out in liver microsomes and in aqueous model systems indicate that the hydroxymethyl groups of the buffer substance Tris can be oxidized to yield formaldehyde and that an oxidant formed via a Fenton-type reaction may contribute to this conversion process. Table 1 demonstrates that formaldehyde is produced from Tris in the Udenfriend system and in rat liver microsomes. Another compound containing hydroxymethyl groups, glycerol, is also converted to formaldehyde in both systems, though less efficiently than Tris. The formation of formaldehyde from glycerol by rat liver microsomes has recently been reported.[10] Bovine liver catalase (6,500 U/ml), propyl gallate (5 μM) and the iron chelator, deferoxamine mesylate (5 μM), suppress formaldehyde formation in the Udenfriend system by 80%, 95% and 90%, respectively. A xanthine oxidase system (0.2 mM hypoxanthine, 70 mU/ml xanthine oxidase) also converts Tris to formaldehyde if Fe(II)EDTA is present. At 300 mM Tris, 0.1 (1.0) nmoles formaldehyde $\times$ ml^{-1} $\times$ min^{-1} are produced with 167 μM (4 mM) Fe(II)EDTA. Catalase (26,000 U/ml) inhibits formaldehyde production by the xanthine oxidase system by 40–50%.

Figure 1 demonstrates the essential kinetic parameters for microsomal Tris oxidation in a Lineweaver Burk plot. Intact NADPH-consuming microsomes are required for the reaction. Table 2 summarizes the evidence obtained for the involvement of an oxidant formed in a Fenton-type reaction in microsomal formaldehyde production from Tris and glycerol. Inhibition due to the omission of the catalase inhibitor, azide, suggests that H_2O_2 or a species derived from H_2O_2 is required. Inhibition is only partial, and exogenous catalase does not add to the effect obtained by the omission of azide. The iron chelator deferoxamine mesylate also suppresses the reaction while addition of Fenton-reactive iron activates formaldehyde production. In the absence of microsomes, Fe (II) EDTA does not lead to the production of formaldehyde from Tris. Oxidation by cytochrome P-450 does not appear to be crucial for formaldehyde production as judged from the lack of inhibition by the monooxygenase inhibitor metyrapone.

TABLE I
Formation of formaldehyde from Tris and glycerol in the Udenfriend system and in rat liver microsomes

	Udenfriend system (nmoles $\times$ ml^{-1} $\times$ min^{-1})	microsomes (nmoles $\times$ mg prot.$^{-1}$ $\times$ min^{-1})
Tris 300 mM	4.33 $\pm$ 0.43	2.86 $\pm$ 0.24
glycerol 300 mM	0.97 $\pm$ 0.13	n.d.
500 mM	n.d.	0.49 $\pm$ 0.02

Values are means $\pm$ S.E. (n = 4 – 28).

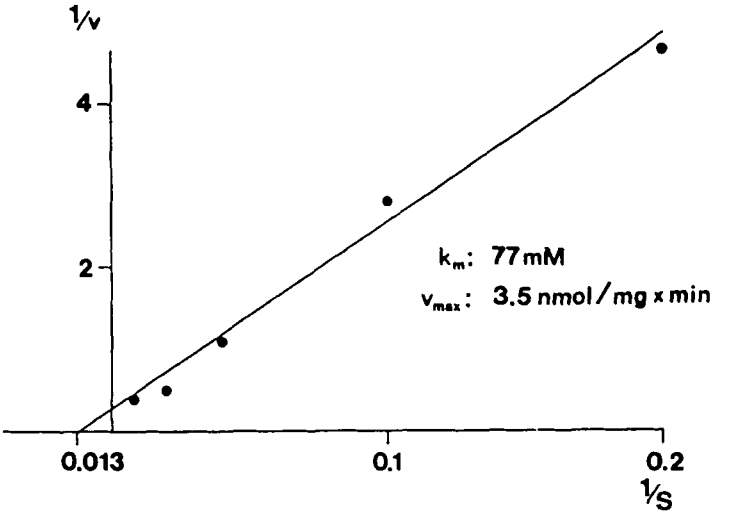

FIGURE 1 Effect of Tris on formaldehyde formation in rat liver microsomes (Lineweaver-Burk plot). v: reaction velocity (nmoles × mg protein^{-1} × min^{-1}); S: Tris concentration (mM). Data are means of 3–4 experiments.

Figure 2 investigates the suitability of the reaction as a test for an oxidant species possessing properties similar to OH· in liver microsomes by means of comparison with an established test, the formation of ethylene from methional.[7] The basal rate of product formation as shown on the ordinate is virtually identical in both tests. Moreover, the influence of two synthetic antioxidants is identical: propyl gallate is a highly active inhibitor in both tests while butylated hydroxytoluene is inactive.

Figure 3 shows that Tris is converted to CO_2 by freshly isolated hepatocytes. CO_2 formation is maximal between 20 and 30 min (about 0.006% of the dose/min) and declines steadily during the following 30 min. Viability decreases slightly during this period. CO_2 formation is markedly suppressed by propyl gallate and by the iron chelator o-phenanthroline (Figure 3). Since the possibility exists that the two inhibitors attack a step in CO_2 production subsequent to the formation of formaldehyde,

TABLE II
Modulation of formaldehyde production from Tris and glycerol in rat liver microsomes

	% of control activity	
	Tris 300 mM	Glycerol 500 mM
+ catalase (bovine liver; 26,000 U/ml), - azide	37 ± 5	37
− azide	43 ± 4	37
+ deferoxamine mesylate (10 μM)	34 ± 4	43
+ Fe (II) EDTA (4 mM)	413 ± 17	227
+ metyrapone (100 μM)	93 ± 11	100

Values are means ± S.E. (n = 3) or means of two experiments.

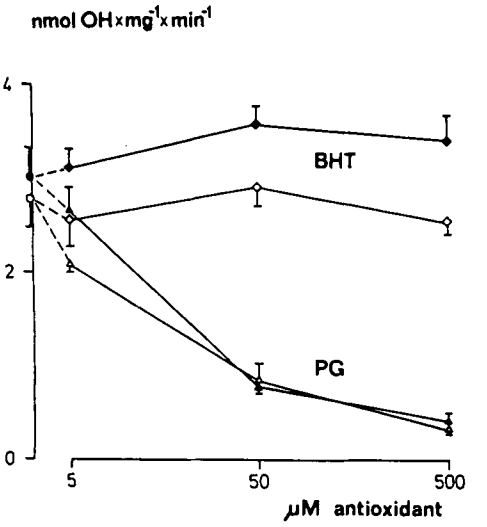

FIGURE 2 Influence of the antioxidants BHT and propyl gallate on hydroxyl radical production in rat liver microsomes: Comparison of Tris test and methional test. Values are means ± S.E.M. (n = 3). Open symbols: ethylene formation from methional; closed symbols: formaldehyde formation from Tris. Inhibition at 50 and 500 μM propyl gallate, $p < 0.001$ against control in both tests.

we excluded that the suppression was due to an inhibitory action of propyl gallate and o-phenanthroline on CO_2 formation from formaldehyde (CO_2 formation at 30 min with 750 μM propyl gallate: 90% of control; with 2 mM o-phenanthroline: 139% of control; n = 4). This indicates that their action in Figure 3 can be localized at the primary oxidation step, formaldehyde formation.

Another OH· scavenger, dimethylsulfoxide, was only moderately active at very high concentrations (40% inhibition at 175 mM). The reason for this is not clear. The iron chelator deferoxamine mesylate which had been active in suppressing formaldehyde formation from Tris in the Udenfriend system and in microsomes was inactive towards CO_2 formation in hepatocytes. We assume that intracellular availability of this agent is insufficient. The presence in the incubation medium of a xanthine oxidase system (0.2 mM hypoxanthine, 70 mU xanthine oxidase/ml) containing 222 μM Fe (II) EDTA enhances the formation of CO_2 from Tris by 67% during the first 5 min of incubation. The enhancing effect slows down rapidly and is no longer observed after 20 min.

[^{14}C] CO_2 is exhaled after the administration of [^{14}C] Tris to rats. Figure 4a shows an experiment with a bolus injection of 2 μCi (74 kBq). Peak exhalation occurs after 10–15 min and amounts to about 0.004% of the dose/min. Experiments have also been carried out with 3.5 μCi (129.5 kBq) and 5 μCi (185 kBq). Maximal exhalation rate expressed as % of the dose/min was approximately constant within this dose range (not shown). In a series of 4 experiments of this type, 0.12 ± 0.02% of the dose had been exhaled after 120 min. Figure 4b demonstrates steady state exhalation in 3 experiments during infusion of 0.5 μCi (18.5 kBq)/h. From the exhalation half lives calculated from bolus injection experiments (45–60 min), it was obvious that steady state exhalation would only be achieved after 3–4 h. Therefore, a primed infusion was performed. Under these conditions, steady state exhalation can be observed in the

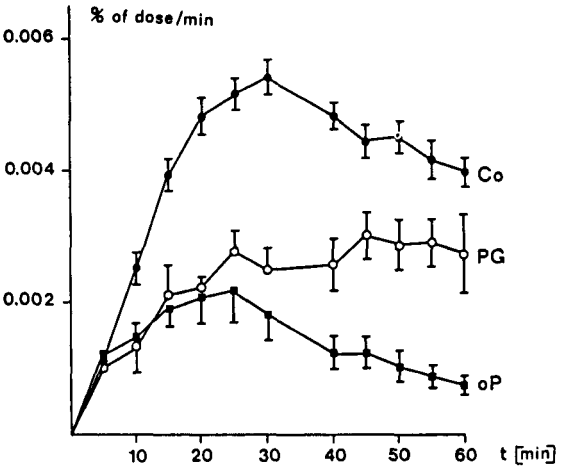

FIGURE 3 Formation of [^{14}C]CO_2 from [^{14}C]Tris by freshly isolated hepatocytes. Tris 2.5 μCi (92.5 kBq) = 0.15 mM. Values are means ± S.D. (n = 3–12). sampling intervals, 5 min; Co: control; PG: propyl gallate, 750 μM; oP: o-phenanthroline, 2 mM. Inhibition was statistically significant (Student's t-test; $p < 0.05$ or less against control) between 15 and 50 min for PG and between 15 and 60 min for oP.

second hour. At 120 min, exhalation velocity is 0.29 ± 0.02% of the dose. Thus, only a minor fraction of the dose is converted into CO_2.

DISCUSSION

The requirements established for one-carbon compound formation from Tris suggest that an oxidant formed via a Fenton-type reaction is involved. The formation of formaldehyde from Tris may explain the recently published observation that during investigation of microsomal demethylation reactions large increases of apparent enyme activity occur in the presence of ascorbic acid and Tris.[11] The authors assumed that this was due to a chemical interaction between ascorbic acid and Tris in the presence of the Nash reagent. However, from our present findings we would suggest that conversion of Tris to formaldehyde by an oxidant formed in microsomes in increased amounts in the presence of ascorbic acid may also be involved.

It should be noted that the identity of the oxidizing species is not unequivocally established by the procedures used in this study. In microsomes, a radical species derived from Tris has previously been detected.[12] Tris is known to react with OH·,[13,14] with a rate constant[14] of $1{,}1 \times 10^9 \text{l} \cdot \text{mol}^{-1} \cdot \text{sec}^{-1}$. However, in a system allowing for the iron-catalyzed Haber-Weiss reaction, oxidation reactions may also be brought about by a Fe (IV) species.[15] Moreover, in a biological system containing various types of Fenton catalysts, the oxidation of a detector molecule must not necessarily be mediated by "free" OH· but might be due to OH· localized at the iron complex where it was formed, provided the detector molecule is capable of associating to the metal catalyst.[16] In addition, alternative oxidants, e.g. alkoxy radicals, may exist whose formation is favored by the presence of H_2O_2 and iron and which therefore share the inhibition and enhancement pattern by the modulators used in the above

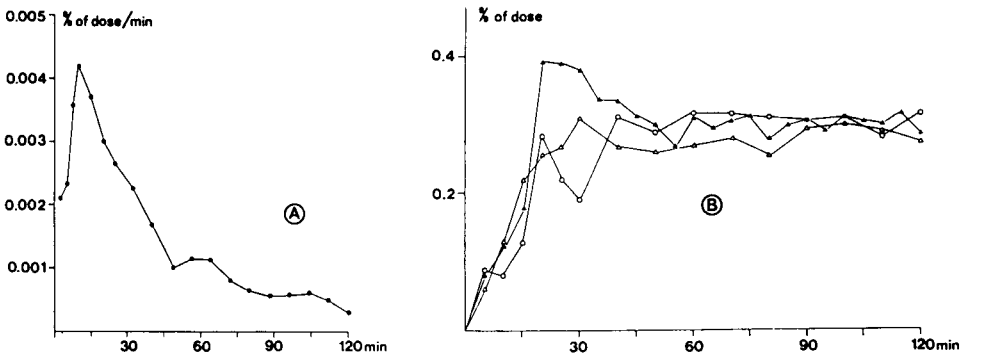

FIGURE 4 Exhalation of [^{14}C] CO_2 by rats following the administration of [^{14}C] Tris. A. Intravenous bolus injection of 2 μCi (74 kBq) = 1 mmol Tris. B. Intravenous infusion. Initial infusion velocity (0–15 min) 2.5 μCi (92.5 kBq)/h = 125 μmol/h. Maintenance infusion velocity (15–20 min) 0.5 μCi (18.5 kBq)/h = 25 μmol/h. Single experiments are plotted. Sampling intervals 8 min (A) or 5 min (B).

experiments. The "calibration" method used in our microsome experiments, ethylene production from methional, has been shown to be nonspecific for OH·.[17] Also, complete inhibition by catalase was not achieved in microsomes and in the Udenfriend system. It is conceivable that various oxidants participate in Tris oxidation.

While most CO_2 formation from formaldehyde can be assumed to occur enzymatically within the cell, the preceding formaldehyde formation from Tris in our hepatocyte experiments may theoretically have taken place both in the medium and in the cell. Since the medium does not produce sufficient amounts of formaldehyde from Tris in the absence of hepatocytes, it must be assumed that in the experiments with propyl gallate and o-phenanthroline, Tris is oxidized within the hepatocyte. Animal experiments on the volume of distribution have suggested that Tris has access to total body water and thus is capable of invading the intracellular space.[4] Considerable doubt as to the alleged rapid penetration of Tris through the cell membrane has recently been raised.[2,18–20] However, the liver cell appears to be an exception to this. Rothe and Heisler have shown that Tris diffuses almost immediately into liver tissue, with 50% equilibration obtained after 3 min.[20] It can therefore be assumed that CO_2 production under control conditions and in the presence of antioxidants reflects the concentration of the oxidant generated within the cell. In the hepatocyte experiments with xanthine oxidase in the presence of FE(II)EDTA, the primary oxidation step leading to the formation of formaldehyde may in part have taken place in the extracellular space. Alternatively, H_2O_2 produced by the xanthine oxidase reaction may have entered the cell and an oxidant formed from it intracellularly may have mediated Tris oxidation within the hepatocyte.

If oxidation by a Fenton product, possibly OH·, plays a major role in CO_2 formation from Tris *in vivo*, this would provide a potential breath test for conditions of oxidative stress. In vivo detection of highly damaging species derived from H_2O_2 is currently a major problem in the field of oxidative stress.[21] Initiation of an oxidative stress in the whole animal might be envisaged to increase CO_2 production from Tris. We have at present no evidence that this does indeed occur. Oxidative stress will cause glutathione depletion; on the other hand, glutathione is required for the metabolism of formaldehyde to formic acid.[22] It is conceivable that this will interfere with the

detection of increased radical oxidant formation by means of CO_2 formation. We intend to study the applicability of the Tris test for the detection of an oxidative stress under *in vivo* conditions in an ischemia/reperfusion model in the rat.

Acknowledgements

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References

1. G.G. Nahas (1962) The pharmacology of tris (hydroxymethyl) aminomethane (THAM). *Pharmacological Reviews* **14**, 447–472.
2. H. Brasch, E. Thies and H. Iven (1982) Pharmacokinetics of TRIS-(hydroxymethyl)-aminomethane in healthy subjects and in patients with metabolic acidosis. *European Journal of Clinical Pharmacology* **22**, 257–264.
3. H.N. Christensen and J. Clifford (1962) Test for metabolic attack on tris (hydroxymethyl)-aminomethane and α-hydroxymethylserine in the rat. *Proceedings of the Society for Experimental Biology and Medicine* **111**, 140–143.
4. M.H. Holmdahl and G.G. Nahas (1962) Volume of distribution of ^{14}C-labeled tris (hydroxymethyl) aminomethane. *American Journal of Physiology* **202**, 1011–1014.
5. T. Nash (1953) The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. *Biochemical Journal* **55**, 416–421.
6. H. Schütte, J. Flossdorf, H. Sahm and M.-R. Kula (1976) Purification and properties of formaldehyde dehydrogenase and formate dehydrogenase from Candida boidinii. *European Journal of Biochemistry* **62**, 151–160.
7. I. Mahmutoglu and H. Kappus (1985) Oxy radical formation during redox cycling of the bleomycin-iron (III) complex by NADPH-cytochrome P–450 reductase. *Biochemical Pharmacology* **34**, 3091–3094.
8. O. Seglen (1976) Preparation of isolated rat liver cells. *Methods in Cell Biology* **13**, 29–83.
9. B.H. Lauterburg and J. Bircher (1976) Expiratory measurement of maximal aminopyrine demethylatin in vivo: Effect of phenobarbital, partial hepatectomy, portacaval shunt and bile duct ligation in the rat. *Journal of Pharmacology and Experimental Therapeutics* **196**, 501–509.
10. D.K. Winters, L.A. Clejan and A.I. Cederbaum (1988) Oxidation of glycerol to formaldehyde by rat liver microsomes. *Biochemical and Biophysical Research Communications* **153**, 612–617.
11. L. Zychlinski, P. Raska-Emery and M.R. Montgomery (1988) Artifacts in the determination of microsomal xenobiotic N-demethylation in the presence of ascorbic acid, Tris buffer and Nash reagent. *Xenobiotica* **18**, 485–490.
12. A.N. Saprin and L.H. Piette (1977) Spin trapping and its application in the study of lipid peroxidation and free radical production with liver microsomes. *Archives of Biochemistry and Biophysies* **180**, 480–492.
13. W. Paschen and U. Weser (1975) Problems concerning the biochemical action of superoxide dismutase (erythrocuprein). *Hoppe-Seyler's Zeitschrift für Physiologische Chemie* **356**, 727–737.
14. M. Hicks and J.M. Gebicki (1986) Rate constants for reaction of hydroxyl radicals with Tris, Tricine and Hepes buffer. *FEBS Letters* **199**, 92–94.
15. H.C. Sutton and C.C. Winterbourn (1989) On the participation of higher oxidation states of iron and copper in Fenton reactions. *Free Radicals in Biology and Medicine* **6**, 53–60.
16. J.M.C. Gutteridge, I.-Z. Nagy, L. Maidt and R.A. Floyd (1990) ADP-iron as a Fenton reactant: Radical reactions detected by spin trapping, hydrogen abstraction, and aromatic hydroxylation. *Archives of Biochemistry and Biophysics* **227**, 422–428.
17. W.A. Pryor and R.H. Tang (1978) Ethylene formation from methional. *Biochemical and Biophysical Research Communications* **81**, 498–503.
18. H. Brasch and H. Iven (1981) Pharmacokinetics of TRIS (hydroxymethyl) aminomethane in the rabbit. *Archives internationales de Pharmacodynamie et de Therapie* **254**, 4–12.
19. K.F. Rothe and N. Heisler (1985) Effect of sodium bicarbonate and tris (THAM) infusion on intra- and extracellular acid-base status during experimental uremia in the rat. *Urologia internationalis* **40**, 190–195.

20. K.F. Rothe and N. Heisler (1986) Distribution of tris buffer between intracellular and extracellular space as a function of plasma pH in the rat. *Acta Anaesthesiologica Scandinavica* **30**, 111–115.
21. B. Halliwell and M. Grootveld (1987) The measurement of free radical reactions in humans. Some thoughts for further experimentation. *FEBS Letters* **213**, 9–14.
22. E. Dicker and A.I. Cederbaum (1986) Inhibition of the low-K_m mitochondrial aldehyde dehydrogenase by diethyl maleate and phorone *in vivo* and *in vitro*. *Biochemical Journal* **24**, 821–827.

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