

## METHYLENE BLUE AS AN INHIBITOR OF SUPEROXIDE GENERATION BY XANTHINE OXIDASE

### A POTENTIAL NEW DRUG FOR THE ATTENUATION OF ISCHEMIA/REPERFUSION INJURY

STEVEN C. SALARIS, CHARLES F. BABBS\* and WILLIAM D. VOORHEES III

Hillenbrand Biomedical Engineering Center and Department of Veterinary Physiology and Pharmacology, Purdue University, West Lafayette, IN 47907, U.S.A.

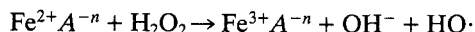
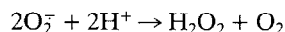
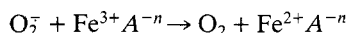
(Received 26 March 1990; accepted 11 February 1991)

**Abstract**—Tissue oxidases, especially xanthine oxidase, have been proposed as primary sources of toxic oxygen radicals in many experimental models of disease states. Among these, ischemia-reperfusion injury may be of the greatest clinical interest. In this paper we propose the use of methylene blue as a means of suppressing the production of superoxide radicals  $O_2^-$  by acting as an alternative electron acceptor for xanthine oxidase. Previous work has indicated that methylene blue accepts electrons from xanthine oxidase at the iron-sulfur center. Initial experiments in our laboratory demonstrated that (1) pairs of electrons from each enzymatic oxidation are transferred to methylene blue, (2) the reduction of methylene blue can be achieved by model iron-sulfur centers, similar to the iron-sulfur center of xanthine oxidase, (3) reduced methylene blue auto-oxidizes to produce  $H_2O_2$  directly, rather than  $O_2^-$ , and (4) methylene blue is effective at non-toxic levels (2–5 mg/kg) in preventing free radical damage to liver and kidney tissues in an *in vitro* model of ischemia and reoxygenation. Accordingly, we propose that methylene blue may represent a new class of antioxidant drugs that competitively inhibit reduction of molecular oxygen to superoxide by acting as alternative electron acceptors for tissue oxidases. We have termed these agents “parasitic” electron acceptors.

An increasing body of evidence implicates the involvement of oxygen-derived free radicals in the pathophysiology of arthritis, iron toxicity, Parkinson's disease, and a variety of disease states including ischemia followed by reperfusion [1–4]. Superoxide ( $O_2^-$ ) is undoubtedly the most abundant oxygen radical generated *in vivo*. Biological sources of superoxide include activated leukocytes [5], the mitochondrial electron transport chain [6], and tissue oxidases, most notably xanthine oxidase [7–9]. The current model of reperfusion injury [1, 7, 10, 11] states that during the ischemic period, hypoxanthine and xanthine substrates accumulate due to the anaerobic breakdown of ATP. At the same time, there is a measurable increase in the amount of xanthine oxidase in the cell due to a proteolytic conversion of xanthine dehydrogenase to xanthine oxidase [12, 13]. Upon reoxygenation, a burst of superoxide radicals may be formed in certain cell types by the action of xanthine oxidase upon accumulated hypoxanthine and xanthine substrates [7].

In the presence of suitably chelated iron, a fraction of the superoxide generated by biological sources may participate in the superoxide driven Fenton

reaction, in which hydroxyl radical ( $HO\cdot$ ) formation occurs [4, 14, 15]:



where  $A^{-n}$  is a chelator anion rendering iron soluble at physiological pH. Nascent hydroxyl radicals, or perhaps intermediate oxo-iron complexes themselves [16, 17], can react with various proteins, nucleic acids or lipids in the cell [4, 18, 19], initiating chains of uncontrolled free radical reactions that may complicate circulatory shock, arterial thromboembolism, cardiac arrest, and other clinical entities.

There are currently several methods under investigation to prevent or attenuate superoxide and iron-mediated injury. The first is the use of antioxidant enzymes such as superoxide dismutase ( $SOD^\dagger$ ) and catalase which scavenge and break down superoxide and hydrogen peroxide, respectively [20, 21]. Alternatively, iron-chelating compounds such as deferoxamine may inhibit superoxide-initiated injury, presumably by preventing the redox cycling of iron [22, 23]. Another attractive approach may be the use of xanthine oxidase inhibitors [2, 24, 25]. Best known among these inhibitors are the purine analogs, oxypurinol and allopurinol, which bind competitively to the purine binding site of the enzyme, displacing hypoxanthine and xanthine. However, another neglected approach to pharmacologic inhibition of  $O_2^-$  production by xanthine oxidase exists. In 1988, Kelner *et al.* [26] re-examined

\* Correspondence: Charles F. Babbs, M.D., Ph.D., Biomedical Engineering Center, Potter Building, Purdue University, West Lafayette, IN 47907.

† Abbreviations: DMSA, dimercaptosuccinic acid;  $HO\cdot$ , hydroxyl radical; HX, hypoxanthine;  $LD_{50}$ , median lethal dose; MDA-LM, malondialdehyde-like materials; NBT, nitroblue tetrazolium; SOD, superoxide dismutase; and X, xanthine.

the early work of McCord and Fridovich [27] suggesting that methylene blue can act as a willing electron acceptor for xanthine oxidase, which allows for the anaerobic oxidation of purine substrates. They proposed that this readily available and already clinically used compound might inhibit the production of superoxide in postischemic tissues [26] by competing with molecular oxygen at the iron-sulfur centers of xanthine oxidase.

Intigued by Kelner's thoughts and observations, we have begun to study methylene blue as a possible inhibitor of  $O_2^-$  production by xanthine oxidase. In this paper, we present an elaboration of Kelner's original idea concerning the mechanism of action and pharmacology of methylene blue during oxidative stress. We propose that methylene blue may attenuate the pathophysiology of reperfusion injury by two methods. First, if given as a pretreatment (prior to ischemia), it may allow for the anaerobic breakdown of hypoxanthine and xanthine to uric acid and thus attenuate the accumulation of hypoxanthine during ischemia. Second, if given either before ischemia or just before reperfusion, methylene blue may help prevent superoxide formation, since it competes favorably with molecular oxygen for reduction by xanthine oxidase [28]. In this hypothetical role, methylene blue does not act as a classical xanthine oxidase inhibitor, because it does not slow the rate of oxidation of hypoxanthine or xanthine. Indeed, it appears to increase the rate of such reactions in some studies [28]. Instead, methylene blue acts as an inhibitor of oxygen reduction, and may well function as such for other oxidases in addition to xanthine oxidase.

Biochemically, our working hypothesis is that methylene blue is reduced to the colorless leuko form, as a result of the action of xanthine oxidase. In the presence of oxygen, the leukomethylene blue then auto-oxidizes back to ordinary methylene blue with the concurrent formation of hydrogen peroxide directly, rather than superoxide. The transfer of electrons in pairs to methylene blue, rather than singly to oxygen, thus short-circuits the formation of superoxide by the enzyme. Without superoxide, the reduction of  $Fe^{3+}$  to  $Fe^{2+}$  does not occur, and even though hydrogen peroxide is formed, there is not sufficient  $Fe^{2+}$  to allow for either the formation of damaging amounts of  $HO\cdot$  by Fenton's reaction or the formation of ferrous-ferrocyanide complexes capable of initiating lipid peroxidation in the absence of  $HO\cdot$  [16, 17]. The hydrogen peroxide that is formed is then readily detoxified *in vivo* by the action of catalase and peroxidases.

Regarding the products of auto-oxidation of leukomethylene blue, there appears to be two schools of thought, one stating that leukomethylene blue auto-oxidizes to hydrogen peroxide [26, 29] and another stating that leukomethylene blue can auto-oxidize univalently to superoxide [27]. Accordingly, we were especially interested in exploring the major products of leukomethylene blue auto-oxidation. Significant univalent reduction of oxygen by leukomethylene blue to form superoxide would obviate the use of methylene blue to suppress superoxide formation.

Routine use of methylene blue as a tissue stain leaves little doubt as to its ability to penetrate intra-

cellular as well as extracellular fluid compartments. The doses required to achieve the desired therapeutic effect, however, are insufficient to render an animal or patient comically blue in color (pseudocyanosis) and indeed are within the range currently recommended for use in human medicine. For these reasons, we gave serious consideration to the potential use of methylene blue as a drug to suppress superoxide formation by xanthine oxidase and performed a number of simple biochemical experiments to (1) explore the number of electrons transferred during methylene blue reduction, (2) determine if model iron-sulfur centers could reduce methylene blue, (3) measure superoxide production during auto-oxidation of leukomethylene blue, and (4) test the ability of methylene blue to suppress one indicator of oxygen radical injury in a tissue model of ischemia and reperfusion.

## METHODS AND RESULTS

*Stoichiometry of methylene blue reduction.* To further explore the antioxidant potential of methylene blue in the laboratory, we first studied the stoichiometry of electron transfer from xanthine to methylene blue. Others have suggested that for each oxidation step, either from hypoxanthine to xanthine or from xanthine to uric acid, there are two electrons transferred to the enzyme [8, 30]. If, in turn, xanthine oxidase transfers two electrons to methylene blue in each oxidation step, we expect 1 mole of leukomethylene blue to be produced for each mole of xanthine oxidized to uric acid and 2 moles of leukomethylene blue for each mole of hypoxanthine oxidized completely to uric acid.

To test this assertion, we performed some simple spectrophotometric studies. A reaction mixture of 0.05 units/mL of xanthine oxidase was added to a 1.5-mL quartz spectrophotometric cuvet containing 37.4 nmol of methylene blue, and 15 nmol of either xanthine or hypoxanthine in argon-saturated (anoxic) Tris-Ringer buffer\* at pH 7.4. The reaction vessel was suffused with argon and covered to exclude oxygen. The conversion of methylene blue to leukomethylene blue was recorded as a decrease in the absorbance of the solution at 667 nm. Based on the extinction coefficient of methylene blue in Tris-buffer ( $4.9 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ —our value), the amount of methylene blue reduced was calculated. Our measured coefficient is in good agreement with the literature value ( $50,119 \text{ M}^{-1} \text{ cm}^{-1}$ ) [31]. The results (Table 1) indicated that xanthine oxidase supplied with 15 nmol of xanthine reduced  $14.6 \pm 0.3$  nmol of methylene blue (mean  $\pm$  SEM) and 15 nmol of hypoxanthine reduced  $29.1 \pm 0.4$  nmol of methylene blue ( $N = 10$ ). These observations confirm that the reduction of methylene blue is a 2-electron process as previously indicated by Blaedel and Meloche [32]. This observation is important, because if leukomethylene blue is a 2-

\* The Tris-Ringer buffer solution included 4.0 mM KCl, 100 mM NaCl, 2.0 mM  $CaCl_2$ , 40 mM Tris, and 200 mg/dL glucose, pH 7.4. Tris was chosen as a buffer rather than phosphate because of its solubility in the presence of calcium.

Table 1. Methylene blue reduction by xanthine oxidase under anaerobic conditions. The substrate was either xanthine (X, 15 nmol) or hypoxanthine (HX, 15 nmol)

Substrate	Change in absorbance	Methylene blue reduced (nmol)	
		Observed	Observed/Expected
X	0.411	12.5	0.833
X	0.482	14.7	0.980
X	0.472	14.4	0.960
X	0.501	15.3	1.020
X	0.475	14.5	0.967
X	0.494	15.0	1.000
X	0.502	15.3	1.020
X	0.490	14.9	0.993
X	0.464	14.1	0.940
X	0.521	15.9	1.060
HX	0.940	28.7	0.957
HX	0.945	28.8	0.960
HX	0.984	30.0	1.000
HX	0.919	28.0	0.933
HX	0.913	27.8	0.927
HX	0.979	29.9	0.997
HX	1.029	31.4	1.047
HX	0.974	29.7	0.990
HX	0.894	27.3	0.910
HX	0.975	29.7	0.990

electron reduction product of methylene blue, then it is reasonable to expect that two electrons might also be transferred to molecular oxygen when methylene blue auto-oxidizes, thus forming  $H_2O_2$  rather than superoxide, as proposed previously by Kelner *et al.* [26].

**Reaction of model iron-sulfur centers with methylene blue.** The reduction of methylene blue by xanthine oxidase occurs in both the molybdenum free and deflavinated enzyme forms [33, 34]. Therefore, it has been suggested that the point of methylene blue reduction must be at the iron-sulfur center [26]. The native xanthine oxidase enzyme contains two iron-sulfur centers of the 2Fe-2S type [8, 35], having two iron atoms chelated by six sulfur moieties (Fig. 1). To further explore this concept, we sought to determine if a reduced iron-sulfur center can, in fact, reduce methylene blue. In simple aqueous solutions, ferrous iron alone did *not* reduce methylene blue and neither did thiols. Hence, the juxtaposition of iron and sulfur atoms of the iron-sulfur center must be critical. To model a reduced iron-sulfur center, we utilized the chelate of  $Fe^{2+}$  and an excess of dimercaptosuccinic acid (DMSA) in dilute, anoxic (argon-saturated) solutions. The combination of these reagents caused an evident interaction between the ferrous iron and the sulfur groups of DMSA, which formed a colored chelate that ranges from brown to pink, depending on the relative concentrations of the two compounds, and was not formed upon combination of ferrous iron with ordinary succinic acid. This model is less complex than the true structure of the iron-sulfur center, but it still includes an  $Fe^{2+}$  surrounded by at least four sulfurs (Fig. 2). These experiments used 50 nmol/mL of  $Fe^{2+}$  chelated in the presence of 800 nmol/mL of DMSA. At the above concentrations, the chelate formed was pink in color and exhibited an absorbance peak at 360 nm.

## XANTHINE OXIDASE

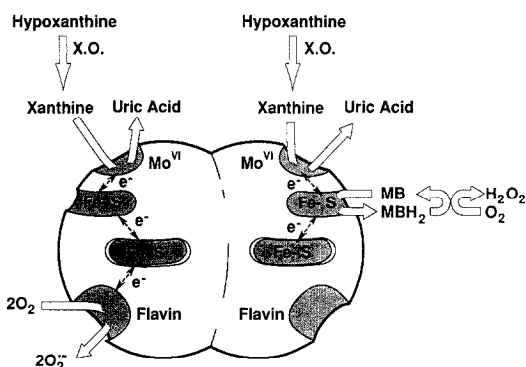


Fig. 1. Cartoon-like sketch of xanthine oxidase, illustrating the proposed role of methylene blue (MB) as a parasitic electron acceptor. The enzyme consists of two subunits, each with purine binding sites,  $Mo^{VI}$ , iron-sulfur (Fe-S) centers, and a flavin [8, 35–37]. The catalytic action is illustrated with molecular oxygen (left) and methylene blue (right) serving as electron acceptors. In the normal sequence on the left, electrons travel to the flavin to be transferred to oxygen, forming superoxide [36, 37]. The authors propose that in the presence of methylene blue, on the right, the electrons are diverted to the dye at the iron-sulfur center. In this way, methylene blue may act as a parasitic electron acceptor that inhibits production of  $O_2^-$ .

In a 1.5-mL spectrophotometer cuvet, 800 nmol of DMSA (from Aldrich) and 50 nmol of  $FeSO_4$  were added to argon-saturated (anoxic) Tris-Ringer buffer (1.0 mL, pH 7.4), after which 15 nmol of methylene blue was added. In  $N = 6$  trials, the absorbance of the methylene blue (667 nm) was seen to decrease

## IRON SULFUR CENTER MODEL

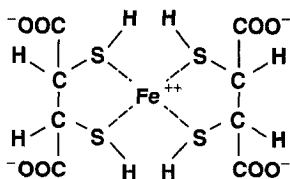


Fig. 2. An idealized configuration of the DMSA-iron chelate. The  $\text{Fe}^{2+}$  is chelated by the four sulfhydryl groups of two dimercaptosuccinic acid molecules. This complex models the iron:sulfur ratio seen in the iron-sulfur centers of xanthine oxidase and appears to mimic the iron-sulfur center of xanthine oxidase in that it is capable of reducing methylene blue.

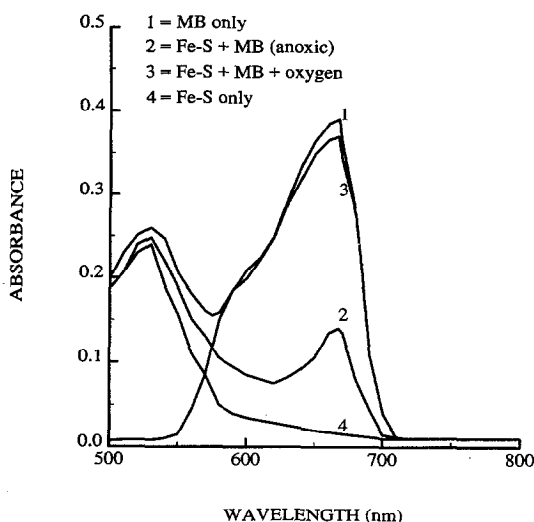
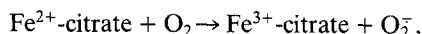


Fig. 3. Typical visible spectra illustrating methylene blue reduction in the presence of the model iron-sulfur (Fe-S) center. (1) A  $6 \mu\text{M}$  concentration of methylene blue (MB) showed characteristic absorbance peak at  $667 \text{ nm}$ . (2) After 3 min, the methylene blue absorbance peak was reduced anaerobically in the presence of the model Fe-S centers ( $50 \mu\text{M}$   $\text{FeSO}_4$  +  $800 \mu\text{M}$  DMSA). (3) When this mixture was reoxygenated, the leukomethylene blue auto-oxidized and the original blue color returned. (4) The spectrum of the chelate alone is included for comparison. Spectra were manually digitized for plotting.  $\text{FeSO}_4$  alone and DMSA alone did not reduce methylene blue (data not shown).

after only 3 min (Fig. 3). The cuvet was then reoxygenated by gently bubbling with 100%  $\text{O}_2$  to promote the auto-oxidation of leukomethylene blue with return of the original color. DMSA-only and  $\text{Fe}^{2+}$ -only controls did not reduce methylene blue ( $N = 3$ ). These simple experiments are consistent with the hypothesis that reduced iron-sulfur centers, similar to those in xanthine oxidase, can, in fact, reduce methylene blue.

**Auto-oxidation of leukomethylene blue.** To more directly test the hypothesis that methylene blue auto-oxidation yields  $\text{H}_2\text{O}_2$  rather than  $\text{O}_2^-$ , we compared auto-oxidation of  $\text{Fe}^{2+}$ -citrate with that of leuko-

methylene blue. Ferrous-citrate is expected to auto-oxidize via a single electron transfer,



thus providing a positive control.  $\text{Fe}^{2+}$ -citrate ( $500 \mu\text{M}$ ) was added to an  $\text{O}_2$  bubbled solution of pH 7.4 Tris buffer. To detect  $\text{O}_2^-$ , nitroblue tetrazolium (NBT,  $500 \mu\text{M}$ ) was added at 0, 15, 30, 45, 60, or 90 sec after  $\text{Fe}^{2+}$ -citrate oxidation had begun and allowed to react for 3 min at room temperature. In the presence of  $\text{O}_2^-$ , NBT is reduced to a purple diformazan, which can be extracted into toluene and measured spectrophotometrically [38]. This approach was selected as an alternative to cytochrome *c* reduction for the present application, because the leuko-dyes as well as  $\text{O}_2^-$  are known to reduce cytochrome *c* [27]. In a similar fashion, methylene blue ( $250 \mu\text{M}$ ) was reduced to leukomethylene blue by addition of equimolar amounts of sodium hydrosulfite at pH 7.4. The solution was then reoxygenated until the blue color completely returned (approximately 10 sec), after which NBT was added at similar times as above. In some experiments, the NBT was added into the leukomethylene blue at time 0 and the solution then reoxygenated. Parallel control experiments were performed in the presence of 2 mg/mL (6000 units/mL) superoxide dismutase from Sigma ( $N = 3$ ).

The reduced NBT from the auto-oxidation experiments was then extracted into 3 vol. of toluene and read in a spectrophotometer ( $\lambda_{\text{max}} = 595 \text{ nm}$ ) against a toluene blank. Based on the extinction coefficient of reduced NBT in toluene (our value  $5.36 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ ), the concentration of reduced NBT was calculated by Beer's Law. Results of such time course experiments comparing auto-oxidation of  $\text{Fe}^{2+}$ -citrate and leukomethylene blue are shown in Fig. 4.

The left panel of Fig. 4 illustrates the SOD inhibitable reduction of NBT following auto-oxidation of  $\text{Fe}^{2+}$ -citrate during the first 150 sec. The decrease in colored diformazan formation with later addition of NBT reflects the spontaneous dismutation of  $\text{O}_2^-$  to  $\text{H}_2\text{O}_2$ . In comparison, the right panel of Fig. 4 shows negligible amounts of SOD inhibitable NBT reduction following auto-oxidation of leukomethylene blue during the first 150 sec. NBT reduction at time 0 is attributable to the direct action of leukomethylene blue in the absence of oxygen, which was not SOD inhibitable. These observations are consistent with the hypothesis that molecular oxygen reacting with leukomethylene blue in buffered, neutral aqueous solution is not reduced to superoxide, but probably undergoes a direct 2-electron reduction to  $\text{H}_2\text{O}_2$ .

**Inhibition of reoxygenation injury in tissue slices.** To test the ability of methylene blue to quench free radical formation in ischemia/reperfusion, we employed a previously described model of ischemia and reoxygenation [39, 40] *in vitro*, in which free-floating liver or kidney slices were exposed to 60 min of ischemia in argon-saturated (anoxic) buffer before being treated with methylene blue at final concentrations of 0, 1, 2, 5, 10 or 25 mg/mL (mg/kg) 10 min prior to reoxygenation. The tissues were then reoxygenated in 100%  $\text{O}_2$  saturated buffer for

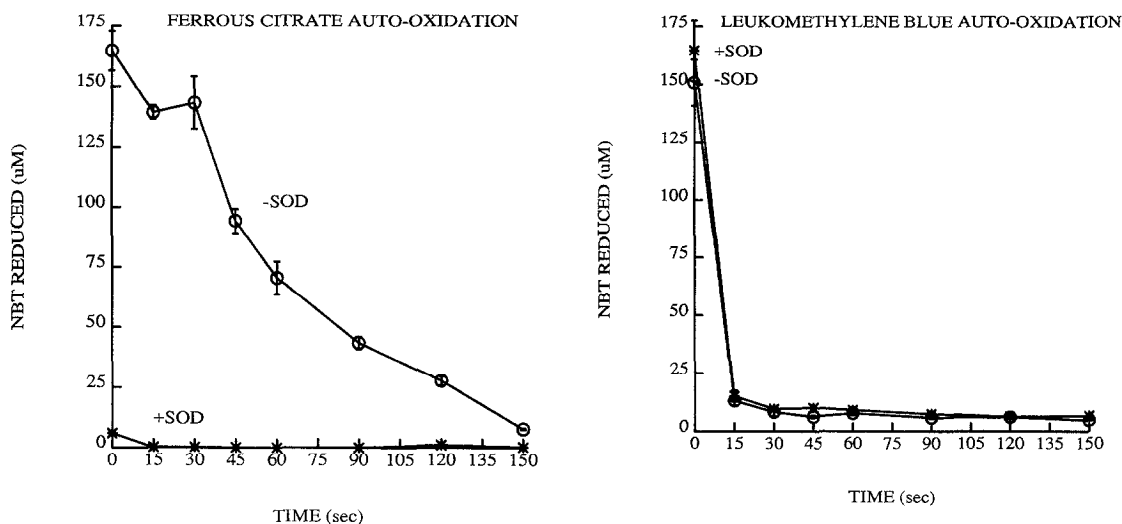


Fig. 4. Left panel: Appearance of SOD inhibitable NBT reducing equivalents after auto-oxidation of  $500 \mu\text{M}$   $\text{Fe}^{2+}$ -citrate in pH 7.4 Tris buffer. Auto-oxidation began at time zero. NBT ( $500 \mu\text{M}$ ) was added at the times indicated and reduced NBT was recovered by toluene extraction and measured spectrophotometrically. The NBT reducing equivalents detected decayed exponentially over time as would be expected for superoxide undergoing spontaneous dismutation. Addition of Sigma SOD (6000 units/mL) completely quenched NBT reduction by this system. This experiment constitutes a positive control since auto-oxidation of ferrous iron chelates is well known to produce superoxide. Values are means  $\pm$  SEM for  $N = 3$ . Right panel: Appearance of NBT reducing equivalents after auto-oxidation of leukomethylene blue. This series of experiments was similar to the series in the left panel. Leukomethylene blue was produced by the addition of  $250 \mu\text{M}$  sodium hydrosulfite to an equimolar amount of methylene blue under anaerobic conditions. The solution was then bubbled with 100% oxygen to initiate auto-oxidation of leukomethylene blue. NBT ( $500 \mu\text{M}$ ) was added at the times indicated, and reduced NBT, recovered by toluene extraction, was measured spectrophotometrically. When NBT was added directly to leukomethylene blue, there was reduction of NBT before addition of oxygen that was not SOD inhibitable. There was no SOD inhibitable NBT reduction after oxidation. Values are means  $\pm$  SEM for  $N = 3$ .

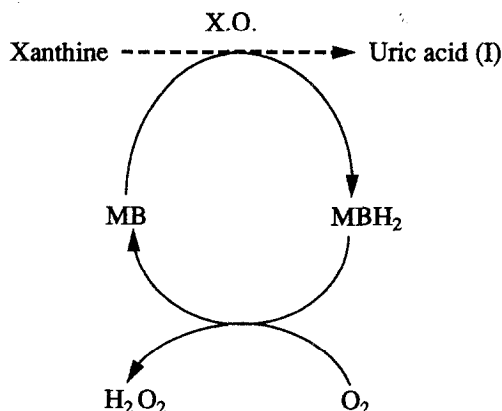
60 min, after which the tissues and buffer were assayed, using the thiobarbituric acid reaction [41], for malondialdehyde-like materials (MDA-LM) which are indicators of free radical mediated lipid peroxidation. The results (Figs. 5 and 6) show that significant production of MDA-LM occurred at zero or low concentrations of methylene blue. However, significant concentration-related attenuation of MDA-LM generation occurred for both tissues. Methylene blue alone did not inhibit the thiobarbituric acid reaction, as was determined by adding various methylene blue concentrations to MDA standards and observing no inhibition of reaction product formation. These data confirm those previously published [39] and suggest that clinically realistic doses ( $5 \text{ mg/kg}$ ) of methylene blue can protect against oxygen radical mediated injury of postischemic tissues.

#### DISCUSSION

The foregoing simple experiments support the claim that methylene blue may well be an effective antioxidant in the setting of ischemia/reperfusion injury when administered in clinically realistic concentrations prior to the return of molecular oxygen. Methylene blue, together with other related compounds such as celestine blue, toluidine blue O, thionin, and azure A, which are also reduced to leuko forms by xanthine oxidase (unpublished obser-

ventions) [42], may constitute a new class of antioxidant drugs that act by suppressing  $O_2^-$  formation by tissue oxidases. In contrast to classical purine analog type xanthine oxidase inhibitors, these agents would not inhibit hypoxanthine and xanthine conversion to uric acid, but would selectively inhibit  $O_2^-$  formation by serving as competitive, alternative co-substrates.

Such alternative electron acceptors seem to be characterized by their apparent ability to accept two electrons, rather than one electron, and, in turn, auto-oxidizing to form  $\text{H}_2\text{O}_2$  directly rather than  $O_2^-$ . That is



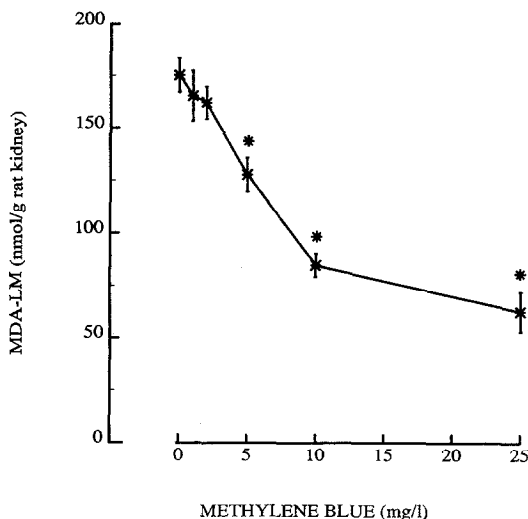


Fig. 5. Postischemic MDA-LM production, an indicator of lipid peroxidation, in rat kidney slices in the presence of various concentrations of methylene blue. Methylene blue was added 10 min prior to reoxygenation of tissues that were ischemic for 1 hr total. Reoxygenation was with 100% oxygen. Mean production of MDA-like materials measured by the thiobarbituric acid method are shown  $\pm$  SEM. An asterisk (\*) indicates significance vs untreated controls at  $\alpha = 0.05$  by Scheffe's multiple comparison test. Values are means  $\pm$  SEM for N = 6.

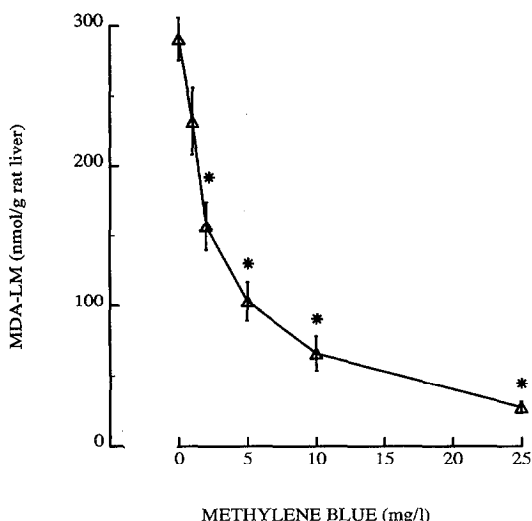
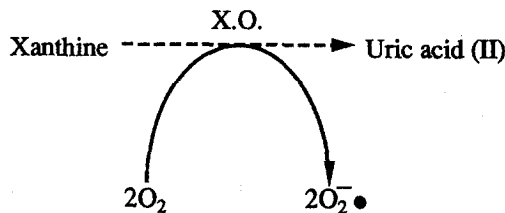


Fig. 6. Postischemic MDA-LM production, an indicator of lipid peroxidation, in rat liver slices in the presence of various concentrations of methylene blue. Methylene blue was added 10 min prior to reoxygenation of tissues that were ischemic for 1 hr total. Reoxygenation was with 100% oxygen. Mean production of MDA-like materials measured by the thiobarbituric acid method are shown  $\pm$  SEM. An asterisk (\*) indicates significance vs untreated controls at  $\alpha = 0.05$  by Scheffe's multiple comparison test. Values are means  $\pm$  SEM for N = 6.

rather than



The high affinity of methylene blue for the iron-sulfur center of xanthine oxidase [28] favors sequence I over sequence II, even in the presence of 100%  $O_2$  during reoxygenation. In this sense, we argue that methylene blue acts as a "parasitic" electron acceptor, shunting electron flow from the normal pathway within the enzyme to form leukomethylene blue at the level of the iron-sulfur centers (Fig. 1, right). The drug effectively short-circuits  $O_2^-$  generation since the electron flow through the enzyme is preferentially routed to methylene blue at the iron-sulfur center rather than to molecular oxygen at the flavin centers. Redox cycling of low molecular weight chelate iron in the superoxide driven Fenton reaction is avoided, because there is no superoxide to reduce  $Fe^{3+}$  to  $Fe^{2+}$ . Although earlier work by McCord and Fridovich [27] indicated that reduced methylene blue was capable of univalently reducing oxygen to superoxide, the detection method used (adenochrome formation) indicated that the rate of superoxide formation was relatively slow. The present results clearly indicate that the formation of hydrogen peroxide, rather than superoxide, is the dominant product of leukomethylene blue auto-oxidation, compared to ferrous citrate auto-oxidation, in which abundant superoxide is formed.

The end result, prevention of  $O_2^-$  generation, is similar to the effect of SOD. However, unlike SOD, methylene blue, a low molecular weight, partially liposoluble agent, is unquestionably able to penetrate cells and tissues, in view of its use as a rapidly penetrating histological stain. These properties may make methylene blue an especially useful pharmacologic agent, which has already been proven safe at likely effective doses in human patients.

Methylene blue is currently used in medicine for the treatment of cyanide poisoning, methemoglobinemia, nitrite poisoning, and urinary tract infections [43–47]. In a guinea pig model of nitrite toxicosis, 10 mg/kg of methylene blue protected against methemoglobinemia following a 50 mg/kg dose of  $NaNO_2$  [47]. As early as 1935, methylene blue was used as a treatment for urinary tuberculosis [44]. The treated human patients showed post-operative weight increase and relief of symptoms in 11 of 12 cases with unilateral renal tuberculosis. In the case of methemoglobinemia in the adult human, methylene blue is given as an injection of 50 mL of a 1% solution. The dye then acts therapeutically to accelerate reductive processes in the cell. It is effective in cases of NADPH-methemoglobin reductase deficiency but not in cases of methemoglobinemia due to glucose-6-phosphate dehydrogenase deficiency [46, 48]. It is also a relatively non-toxic compound and can be used over a wide range of doses. In

humans with cyanide poisoning, a 500 mg bolus is given which corresponds to a dose of approximately 7 mg/kg [49]. In the dog and rat, the LD<sub>50</sub> values (median lethal dose) for i.v. methylene blue are 50 and 1250 mg/kg, respectively. In monkeys, the lowest published toxic dose is 10 mg/kg [50].

The action of methylene blue and similar drugs as parasitic electron acceptors opens up a variety of therapeutic possibilities. As a pretreatment, as in cases of aortic cross-clamping or organ transplantation, methylene blue might be given just prior to the ischemic period to provide a substrate for the anaerobic breakdown of hypoxanthine and xanthine. In conditions such as circulatory shock, arterial thromboembolism, or bowel torsion with strangulation, methylene blue could be given as a post-treatment (i.e. after the onset of ischemia) and still be effective, since the competition kinetics favor the reduction of methylene blue rather than molecular oxygen [28]. The low-toxicity, effectiveness, and unique biochemistry of methylene blue and its congeners, may make such compounds valuable in the future study and treatment of tissue injury thought to be mediated by xanthine oxidase and superoxide.

**Acknowledgements**—This work was supported by Grants HL-36712 and HL-35996 from the National Heart, Lung, and Blood Institute, U.S. Public Health Service, Bethesda, MA.

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