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A Rapid, Widely Applicable Screen for Drugs that Suppress Free Radical Formation in Ischemia/Reperfusion

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ABSTRACT

Substantial injury can occur during reoxygenation of previously ischemic tissue in many experimental models, as the result of the generation of oxygen-derived free radicals. To test the antiradical activity of potentially protective compounds in this setting, we developed a simple screening system, applicable to fresh biopsy specimens, in which warm ischemia and reoxygenation of excised tissue are performed in vitro. Tissue production of malondialdehyde (MDA) equivalents is used as a nonspecific-but-sensitive marker of oxygen radical damage. Test compounds with putative antiradical activity are added prior to the reoxygenation phase, and their ability to suppress MDA production is an index of activity in preventing reoxygenation injury. Comparison with ischemic but not reoxygenated controls confirms the oxygen-dependent nature of the effect. Standard positive controls of known effective agents, such as butylated hydroxytoluene or deferoxamine, provide a reference for the activity of the test compound. The method is applicable to surgical biopsy specimens in veterinary and human medicine.

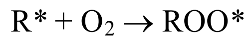
Key words: Superoxide radical; Hydroxyl radical; Oxygen toxicity; Reperfusion; Reoxygenation; Reperfusion injury; Antioxidant; Iron chelator; Thiobarbituric acid; Malondialdehyde

INTRODUCTION

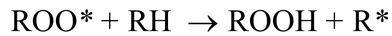
The term reperfusion injury refers to tissue damage observed after a period of ischemia and reperfusion that is manifested, not during the ischemic period, but rather during the reperfusion phase. Such reperfusion injury is to be distinguished from primary ischemic injury, which develops during the ischemic phase ***per se*** and which is halted in its progression by the restoration of tissue oxygenation.

A major mechanism for the genesis of reperfusion injury appears to be the generation of active oxygen species, including superoxide radicals, $(O_2*)^-$, and hydrogen peroxide, H_2O_2, which can be converted in the presence of tissue iron to highly reactive hydroxyl radicals, HO*. (Here we use the symbol * to indicate the unpaired electron of the free radical.) Hydroxyl radicals

initiate deleterious chain reactions by removing hydrogen atoms from biological substrates, $HO^* + RH \rightarrow R^* + H_2O$. In the presence of molecular oxygen, the resultant secondary radicals, R^*, can participate in self-propagating peroxidation reactions



$$R^* + O_2 \rightarrow ROO^*$$



$$ROO^* + RH \rightarrow ROOH + R^*$$

that amplify the injurious effects of previous ischemia at the same time that normal aerobic energy metabolism is being restored (McCord, 1985; Babbs, 1985; Aust et al., 1985; Bulkley, 1983; Nayler and Elz, 1986). Polyunsaturated fatty acids are especially susceptible to peroxidation by such radical chain mechanisms (Tien and Aust, 1982). The resultant lipid hydroperoxides (ROOH) spontaneously rearrange to produce multiple degradation products, including easily measured malondialdehyde (MDA). MDA-like materials can also be formed as a result of peroxidation of sugars, proteins, and nucleic acids (Slater, 1972,1984; Bird, 1984; Gutteridge and Toeg, 1982), making MDA a nonspecific but sensitive marker for oxygen radical damage to tissues.

At present, the relative susceptibilities of various tissues in various species to such reoxygenation injury is not well established. In particular, the relative ability of human tissues to produce free radicals during reoxygenation is not known. Moreover, the durations of ischemia required to set the stage for reperfusion injury in various tissues have not been systematically compared. Accordingly, there is a need for a versatile screening procedure to evaluate both the conditions most favoring reperfusion injury and the effects of putative anti-reperfusion injury drugs in tissues of many species, including man. Comparative aspects are especially important because of the well known differences among species in antioxidant defense systems (Fridovich, 1975,1983; Oberley and Oberley, 1986; Loven et al., 1980) and the large differences between tissues and species in the activity of superoxide-generating enzymes such as xanthine oxidase (Parks and Granger, 1986; Al-Khalidi and Chaglassian, 1965).

Many classes of drugs may offer protection from reperfusion injury, including xanthine oxidase inhibitors, monoamine oxidase inhibitors, iron chelators, superoxide dismutases, copper chelates of salicylates, 21-amino steroids (Braughler et al., 1987), and classical chain-breaking antioxidants such as vitamin E. Only a few such compounds have been evaluated in the published literature of reperfusion injury. In most studies, the compounds were given before ischemia, rather than at the onset of reoxygenation, so that their efficacies in preventing reperfusion injury in particular, as opposed to primary ischemic injury, are not revealed.

We describe a simple screening system applicable to most tissues, in which warm ischemia and reoxygenation of excised tissue specimens are performed in vitro, and production of malondialdehyde (MDA) equivalents during reoxygenation is used as a nonspecific but sensitive marker of oxygen radical damage. Compounds with putative antiradical activity are added prior to the reoxygenation phase, and the ability of test compounds to suppress MDA production is an index of their activity in preventing reoxygenation injury. Comparison with ischemic but not reoxygenated controls confirms the extent to which MDA production occurred during the

reoxygenation phase. Standard positive controls of known effective agents, such as butylated hydroxytoluene or deferoxamine, provide a reference for the activity of the test compound. Nonischemic controls are not required, the harvest of tissue specimens is feasible during standard surgical biopsy procedures, and a wide variety of experimental models.

[Note: Many authors use the term "thiobarbituric acid reactive substances" (TBARS) to indicate that products of tissue oxidation other than malondialdehyde can cause positive reaction in the colorimetric assay for using thiobarbituric acid. For simplicity, we have chosen the term malondialdehyde equivalents--or simply MDA--for use in the present text.]

METHODS

Materials

Butylated hydroxytoluene (BHT), 2-thiobarbituric acid, and TRIS buffer were obtained from Sigma Chemical Company (St. Louis MO, USA), trichloracetic acid from Fisher Scientific Company (Fair Lawn, NJ, USA), and methylene blue from Aldrich Chemical Company (Milwaukee, WI, USA). Deferoxamine, Lot # 30315, was kindly provided by Ciba-Geigy Pharmaceuticals Division (Suffern, NY, USA). 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 buffer was chosen because its solubility in the presence of calcium permitted a greater buffering capacity to neutralize lactic acid produced by tissues during ischemia than the usual phosphate buffers.

Animals

In these initial studies, tissue samples were taken from 10 male Wistar rats anesthetized with sodium pentobarbital (60 mg/kg, i.p.). Liver, kidney, and heart were isolated and divided into five samples of approximately 0.4 g (0.2 g for heart), which were weighed and transferred to stoppered 50-ml Erlenmeyer flasks containing argon-saturated (anoxic) TRIS-Ringer solution.

Ischemic anoxia/Reoxygenation in Short-Term Organ Culture

The 50-ml Erlenmeyer flasks, containing tissue samples and 3.9 ml of argonated TRIS-Ringer buffer, were flushed with argon gas, restoppered, and kept in a warmed, 37°C water bath without shaking for 50 min to create ischemic anoxia. Mincing of the tissue and shaking were avoided at this stage to minimize diffusion of metabolites from the tissue into the medium that would not occur during ischemia in vivo.

Mincing and Drug Addition

After 50 min of anoxia, the tissues were minced with iris scissors into fragments approximately 1 mm on a side. Because argon is more dense than air, its displacement from the flasks and the substitution of oxygen were minimal during mincing and subsequent drug addition. Then, 0.1 ml of TRIS Ringer buffer containing one of several antioxidant drugs to be evaluated was added to

achieve the desired final concentrations: deferoxamine, 100 mg/L; methylene blue, 10 mg/L; butylated hydroxytoluene (BHT), 150 mg/L (initially dissolved in ethanol); or nondrug-containing control buffer. Deferoxamine was selected as a prototypic iron chelator, able to prevent iron catalyzed HO* production (Gutteridge et al., 1979). Methylene blue was selected as a xanthine oxidase inhibitor to block superoxide production by xanthine oxidase (Fridovich and Handler, 1962). BHT was selected as a classical antioxidant, i.e., a compound that interferes with radical chain propagation. These additions were made quickly to avoid introduction of oxygen. The flasks were restoppered, swirled gently to mix the tissues with drugs, and returned to the water bath for a 10-min equilibration period prior to reoxygenation.

Reoxygenation

After 60 min of ischemic anoxia, the flasks were reoxygenated by vigorous bubbling of 100% oxygen gas into the samples for 1 min and flushing of the headspace with oxygen. (Argon gas was substituted for oxygen at this stage in the ischemic but not reoxygenated control experiments.) All flasks were then stoppered and returned to the water bath, with shaking, for 60 min of reoxygenation at 37°C. This method is described as a "free floating" slice culture (Dickson and Suzangar, 1976), the tissue fragments being suspended without support in the medium. The surface area of the medium is approximately 12 cm^2, and its depth is only about 3 mm, to minimize time for equilibration of the medium with headspace gas.

Thiobarbituric Acid Assay for Malodialdehyde

Malondialdehyde was measured by the spectrophotometric method of Buege and Aust (1978). Flask contents, including tissue and buffer, were homogenized in a Teflon/glass homogenizer. The total homogenate was combined with 4.0 ml of TCA/TBA reagent (Buege and Aust, 1978) in a test tube and mixed thoroughly. Each tube was covered with a glass marble and heated for 15 min in a water bath at 90-95°C. After cooling, the flocculent precipitate was removed by centrifugation at 1500 g for 5 min. The absorbance of the supernatant was determined spectrophotometrically at 500, 530, and 560 nm against a blank containing 4 ml of TCA/TBA reagent, 3.9 ml buffer, and 0.1 ml of the corresponding drug or buffer. Standard curves were prepared using authentic malonaldehyde (bis dimethyl acetal) [1,1,3,3-tetramethoxypropanol, available from Aldrich Chemical Company, Inc. (Milwaukee, WI, USA, Cat. #10,838-3).

Data Analysis

We developed a new, more specific double derivative technique to calculate the concentration of MDA equivalents, which is a modification of the standard assay that eliminates most of the nonspecific background interference present in normal plasma or tissue samples. The concentration of MDA equivalents in the spectrophotometer cuvet was computed as

$$c = \frac{2A_{530} - A_{500} - A_{560}}{(2 - b_{500} - b_{560})\,\epsilon\, d}$$

where subscripts represent wavelengths in nanometers, A is absorbance, the constants $b_{500} = A_{500}/A_{530}$ for authentic MDA standard and $b_{560} = A_{560}/A_{530}$ for authentic MDA standard, ε is the molar extinction coefficient for authentic MDA, $1.56 \times 10^5 \ M^{-1}cm^{-1}$, and d is the light-path length through the cuvet. This relationship, derived in the Appendix, provides a measure of concentration based upon the 530- nm peak of genuine MDA, which is insensitive to background interference that does not exhibit a peak at 530 nm.

For each tissue type, heart, liver, or kidney, a one-way analysis of variance (ANOVA) was performed to test the null hypothesis that MDA production was the same in the reoxygenated, ischemic-anoxic, and drug-treated tissues. The ANOVA was preceded by a Bartlett's chi-square test for homogeneity of variance (Cooper, 1969). If the variances of the MDA values, y, in the treatment groups were not similar, a suitable transformation was found of the form, $y' = Ay/(K + y)$ for constants A and K, and the ANOVA performed on the transformed data (Anderson, 1974). Specific comparisons of the treatment groups were made using a Scheffe multiple-comparison test. A p value of 0.01 was considered significant.

RESULTS

To illustrate application of the method, Figure 1 presents production of MDA equivalents in various postischemic tissues of the rat. Each series of bars represents a particular tissue. The first bar in each series represents results after 60 min of ischemia followed by 60 min of reoxygenation. The second bar in each series represents results obtained under identical experimental conditions, except for the substitution of argon for oxygen, i.e., ischemic, but not reoxygenated, control tissue. The remaining bars in each group represent MDA production by ischemic tissues reoxygenated in the presence of three prototype antiradical compounds: deferoxamine, methylene blue, and BHT. The effects of various treatments of MDA production were statistically significant for all three tissues ($F > 9$, $p < 0.01$), but much more pronounced for kidney and liver than for heart. In particular, all three tissues produced significantly more MDA with ischemia and reoxygenation than with ischemia alone, and all three tissues produced significantly more MDA in the absence than in the presence of the antioxidant drugs deferoxamine, methylene blue, and BHT ($F > 12$, $p < 0.01$).

Tissue differences in oxygen radical damage, indicated by production of MDA equivalents, are quite striking in this system. Postischemic kidney and liver tissues produce MDA much more abundantly per unit weight than does cardiac muscle. MDA production is significantly inhibited by all three types of antiradical drugs, presumed to act by different mechanisms. Evidently, inhibition of superoxide production by methylene blue, chelation of iron by deferoxamine, and interruption of chain propagation by BHT each quench MDA production in this model. Parenthetically, the effects of methylene blue are not attributable to nonspecific, antioxidant effects, since the drug does not inhibit MDA production by liver treated by cumene hydroperoxide, whereas BHT, a classical chain-breaking antioxidant, does (data not shown). Thus, the model appears able to detect a broad spectrum of antiradical activities represented by these prototype compounds.

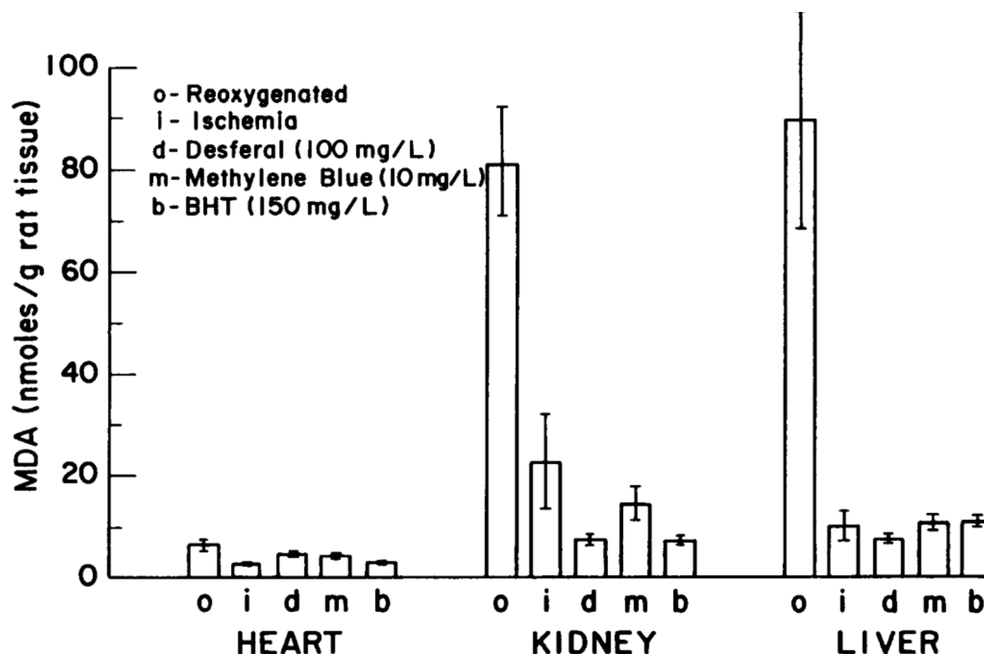


FIGURE 1. Tissue malondialdehyde (MDA), as determined by the thiobarbituric acid method, in excised, postischemic heart, kidney, and liver of the rat. Bars represent mean ± standard error for 10 animals. Abbreviations: o = ischemic and reoxygenated; i = ischemic and not reoxygenated; d = deferoxamine treated 100 mg/L in reoxygenation medium; m = methylene blue treated 10 mg/L in reoxygenation medium; b = BHT treated 150 mg/L in reoxygenation medium.

DISCUSSION

The present paper demonstrates a simple and rapid screening test for oxygen radical production in soft tissues, applicable to a wide variety of biopsy specimens. Differences in radical-mediated tissue damage, measured as postischemic production of MDA equivalents in various tissues of the rat, are readily apparent. Liver and kidney tissues produce abundant MDA per gram after 60 min of ischemia; cardiac muscle produces comparatively little. The use of an ischemic but not reoxygenated control, rather than a nonischemic control, allows application of the technique to fresh biopsy specimens obtained at surgery from diverse species, including man. Tissues can be harvested at surgery by procedures that induce up to several minutes of warm ischemia without spoiling the results, since all tissues in a typical experiment are rendered ischemic for 60 min.

In interpreting the results of such experiments, it is helpful to clarify the distinction between what White et al. have called ischemic hypoxia versus hypoxic hypoxia (White et al., 1984), as well as the terms reperfusion and reoxygenation. Ischemia hypoxia is hypoxia without capillary perfusion. Hypoxic hypoxia is hypoxia with perfusion, as can occur in asphyxiation or in experimental perfusion of isolated organs with oxygen poor solutions, as in the classic

experiments of Hearse et al. (1973). In this latter type of experiment, it is possible that continued clearance of carbon dioxide, extracellular potassium, etc., may create an environment different than that of true ischemia. Similarly, we may say that the term reperfusion refers strictly to reoxygenation with capillary perfusion, whereas reoxygenation may include oxygen delivery without capillary perfusion.

The present model includes genuine ischemic hypoxia (without blood flow) at 37°C. The purpose of argon saturation of flasks during the ischemic phase is simply to avoid oxygen contamination. What is different from the natural situation is that reoxygenation is accomplished by diffusion from the surfaces of tissue fragments, rather than by blood perfusion. By keeping the diameters of the fragments less than 1 mm and the diffusion distance less than 0.5 mm, we hope to minimize the artificiality of this situation. One-half millimeter is the classical diffusion distance applied to tissue slices in organ culture, as derived theoretically and demonstrated practically by Trowell (1952). Our preliminary observation that MDA production by fresh, nonischemic liver tissue slices continuously oxygenated for 1 hr (3.2 ± 2.2 nmol/g, $n = 12$) is equal to or less than that of other controls (Fig. 1) is suggestive of the adequacy of tissue oxygenation in this system, as does the observation of Fraga et al. (1988) that MDA production by 0.5-mm liver slices in response to various oxidants is compatible per cell to that obtained in tissue homogenates or isolated microsomes.

Nevertheless, the term reoxygenation rather than reperfusion should be applied to these experiments, and the results should be interpreted as a model of reperfusion in short term organ culture (Dickson and Suzangar, 1976) rather than reperfusion itself. A corresponding advantage of these flask experiments, however, is that MDA-like materials are not lost from the tissue of origin into the circulation or perfusion medium at rates that are perfusion dependent, as would occur with intact animal or perfused organ experiments.

In a similar sense, the present method is limited in that drug delivery to tissue is by surface absorption rather than via a normal capillary network. Thus, some poorly absorbed drugs may fail to suppress MDA production in this model, yet might still be effective in vivo. We have found this to be the case for allopurinol and its metabolite, oxypurinol, which gave inconsistent results in the present model (data not shown), but which can suppress reperfusion injury when given intravenously to rats and cats (Badylak et al., 1986, 1987; Parks et al., 1982; Toledo-Pereyra, 1974). We attribute this finding to poor penetration of tissue slices by allopurinol in the short-term organ-culture preparations, since the more water-soluble xanthine oxidase inhibitor, methylene blue, did inhibit MDA production in our model. Presumably, drugs that require more than a few minutes to distribute to intracellular tissue compartments may show less than full activity when they are added to flasks after the ischemic phase. A pretreatment experiment, in which the drug is given to the test animal before tissue is harvested for ischemia/reoxygenation, may be necessary in some cases to eliminate this possibility.

Other limitations of the method are related to use of the MDA assay. Although it is sensitive and easy to perform, the MDA assay has a number of well recognized limitations (Slater, 1972,1984; Bird and Draper, 1984). Foremost among these is the ability of compounds other than MDA to produce a positive reaction. MDA-like materials, or so-called thiobarbituric acid reactive

substances, are produced to varying degrees by the action of known oxygen radical generating systems upon biological lipids, proteins, carbohydrates, and nucleic acids (Tien and Aust, 1982; Slater, 1972, 1984; Bird and Draper, 1984; Gutteridge and Toeg, 1982). Most commonly, MDA has been used as an indicator of lipid peroxidation (Tien and Aust, 1982; Slater, 1984). In the present application, we do not interpret MDA as a measure of any specific radical species or the product of any specific radical reaction, but rather as a nonspecific, but sensitive, marker of abnormal oxidation of tissues. The low values obtainable in ischemic, but not reoxygenated, controls attest to the low levels of 530-nm-absorbing species that are not related to reoxygenation injury in some way. Moreover, any nonspecific interference by the commonly encountered yellow species that absorb around 455 nm, rather than at the 530-nm peak of authentic MDA, is minimized by the peak extraction technique described in the Appendix. This modification improves the signal-to-noise ratio of the MDA test in a manner similar to the more laborious methods of Satoh (1978) and Ohkawa et al. (1979), while preserving the simplicity and speed of the Buege and Aust method.

The kinetics of MDA elimination from tissues can also complicate interpretation of some experiments in intact animals. MDA is largely water soluble, with a water: n-octanol partition coefficient of 93:7 (Esterbauer, personal communication) and so is cleared from tissues in vivo by the circulation, as has been readily demonstrated in isolated heart experiments (Gaudel and Duvelleroy, 1984) and in humans, who exhibit raised levels of MDA in the peripheral blood after myocardial infarction (Dousset et al., 1983; Aznar et al., 1983). In the present method, however, total MDA in the reoxygenation flask is measured, so that washout of the material does not occur. Another well recognized characteristic of MDA is its ability to cross-link with plasma proteins and to be otherwise metabolized (Esterbauer et al., 1982). Although some tissue metabolism is inevitable, we minimize this possibility by assaying the samples immediately after the reoxygenation period. Finally, MDA production per gram wet weight may not strictly correlate with relative functional impairment of various tissues after reoxygenation injury, since some tissues may be more resistant to free radical damage than others.

Nevertheless, MDA production by ischemic and reoxygenated biopsy specimens can be used as a useful screening procedure for oxygen radical production to answer limited questions under controlled experimental conditions. As Tappel and coworkers have pointed out (Fraga et al., 1988; Sano et al., 1986), studies of tissue slices in short-term organ culture provide a good compromise between biochemical and physiological systems: They maintain many aspects of whole-tissue organization; many organs can be studied and compared from the same animal, drug, ion, and oxygen concentrations can be precisely controlled; and the preparations are time efficient and less susceptible to manipulation errors than systems employing isolated cells or cell fractions. In the application presented here, we find the method to be useful, not only in comparing the ability of various tissues in various species to produce and be damaged by free radicals, but also in identifying agents capable of protecting against free-radical-mediated injury when it occurs.

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APPENDIX

Slope Difference Method for Peak Extraction

This approach relates the concentration of a light-absorbing compound to the second derivative of the absorbance versus wavelength spectrum, rather than to the absolute value of absorbance. Consider a spectrum of absorbance, A, plotted as a function of wavelength, λ, and having a peak absorbance of interest at wavelength λ_2. Let wavelengths λ_1, λ_2, and λ_3 represent equally spaced points along the wavelength axis, centered on the absorbance peak at λ_2. Let constants b_1 and b_3 represent the fractions of the peak absorbance of the authentic spectrum of a known standard (std) at wavelengths λ_1 and λ_3, namely $A_{1,std} = b_1 A_{2,std}$ and $A_{3,std} = b_3 A_{2,std}$. Let $\Delta\lambda = \lambda_2 - \lambda_1 = \lambda_3 - \lambda_2$. The slope change of the true absorbance spectrum of the sample near the peak may be approximated as

$$\frac{A_2 - A_1}{\Delta\lambda} - \frac{A_3 - A_2}{\Delta\lambda} = \frac{A_2}{\Delta\lambda}(2 - b_1 - b_3)$$

and related to concentration by substituting Beer's Law, $A_2 = \varepsilon cd$, where ε is the molar absorptivity, c is the concentration of absorber, and d is the light path length. In this case

$$\frac{A_2 - A_1}{\Delta\lambda} - \frac{A_3 - A_2}{\Delta\lambda} = \frac{\epsilon cd}{\Delta\lambda}(2 - b_1 - b_3)$$

or

$$c = \frac{2A_2 - A_1 - A_3}{(2 - b_1 - b_3)\,\epsilon d}\,.$$

This expression is a practical computational formula.

Because concentration is calculated from the slope difference about the peak rather than the absolute value, it is clear (and easily shown formally) that the result is unaffected by interfering absorbance that is either essentially constant or linearly changing over the range λ_1, to λ_3. Indeed, addition of spectral interference that is nonlinear, but which exhibits three points at wavelengths λ_1, λ_2, and λ_3 that happen to fail on a straight line, will not affect the result. Nonspecific tissue interference, e.g., nonischemic tissue blanks or normal blood plasma blanks, have exactly these spectral features (low and flat without peaks). These interferences are effectively removed by the slope difference method.

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