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Visible light induced lipoperoxidation of a parenteral nutrition fat emulsion sensitized by flavins

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The photosensitizing effects of riboflavin, flavin mononucleotide, flavin adenine dinucleotide, and a commercial multivitamin infusate on a lipid emulsion used in parenteral nutrient infusions were studied during exposure to polychromatic visible light. It was found that the efficiency of riboflavin (a polar flavin) as sensitizer of lipid emulsion peroxidation is greater than that of flavin mononucleotide (an ionic flavin). This was determined by measuring the consumption of molecular oxygen and the concentration of thiobarbituric acid-reactive species generated during the irradiation time. These findings are supported by molecular orbital studies of these molecules related to polarity, ionic charges around the different molecular regions, and electrostatic potentials comparisons. Flavin adenine dinucleotide (a more ionic flavin), most likely remains totally excluded from the lipid emulsion due to its polarity and molecular geometry and does not induce lipid peroxidation. The multivitamine complex seems to provide a protective effect on the lipid emulsion exposed to light, attributed to the presence of ascorbic acid, which suffers an intensive photodecomposition. A solution of vitamin C, whose concentration was equivalent to that of the parenteral mixture, consumes a considerable amount of molecular oxygen when it is irradiated with visible light in the presence of flavin mononucleotide. (J. Nutr. Biochem. 9: 149–154, 1998) © *Elsevier Science Inc. 1998*

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Introduction

Commercially available fat emulsions are widely used clinically as a component of parenteral nutrients. It has been proposed that the emulsion droplets may have a multilayered surfactant arrangement as well as an inclusion of water vesicles in the oil phase of the emulsion.[1] The lipid emulsions for total parenteral nutrition contain large amounts of polyunsaturated fatty acids (PUFAs) of the (n-3) series. This large unsaturation of lipid emulsion make them sensitive to peroxidative processes. In a completely peroxide-free lipid system, initiation of a peroxidation sequence in a PUFA is the attack of any species that has sufficient reactivity to abstract a hydrogen atom from a methylene group adjacent to a double bond.[2] Vitamin B_2, present in the multivitamin infusate which is added to the fat emulsion during a total parenteral nutrition, has undesireable properties as a photochemical sensitizer. It leads to substrate oxidation through both Type I and Type II mechanisms[3,4] in the presence of light and molecular oxygen. In the Type I mechanism, the substrate initially reacts with the sensitizer in the triplet state and then with molecular oxygen (or one of its active species) via a radical intermediate. In the Type II mechanism, the excitation energy is transferred from the sensitizer in the triplet state to molecular oxygen, giving rise to $^1O_2$, which in turn, reacts with the substrate. If the substrate concentration is higher than that of the molecular oxygen the Type I mechanism prevails.

In this paper the photoinduced peroxidative capacity of flavins was studied, including that of flavin mononucleotide, normally present in parenteral nutrients. It is postulated that although the reduction potential of riboflavin is −0,3 V at pH 7.0,[5] the redox potential of the triplet flavin can be considered to be shifted to 1.4 V,[6] which could be high enough to abstract a hydrogen atom from polyunsaturated lipids and initiate a peroxidative-reaction sequence.

Molecular orbital studies were performed to characterize the geometry, electrostatic potentials, frontier orbital prop-

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erties, and charge distributions located at the different molecular regions for riboflavin (RF), flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD). We show that these theoretical calculations support our experimental results.

Methods and materials

Ascorbic acid, FAD, FMN, malonaldehyde bis(dimethyl acetal), RF and 2-thiobarbituric acid were from Sigma Chemical Company (St. Louis, MO USA). All other reagents were analytical grade. The two ampoules (MVI-1 and MVI-2) that contain the multivitamin infusate added to daily parenteral nutrition admixtures were obtained from Rhone Poulenc Rorer (Collegeville, PA, USA). MVI-1 (5 mL) has the following composition: 100 mg vitamin C, 0.99 mg vitamin A, 5.0 μg vitamin D, 3.0 mg thiamine, 3.6 mg FMN, 4.0 mg vitamin B_6, 40 mg niacinamide, 15 mg pantotenic acid, and 10 mg vitamin E. MVI-2 (5 mL) has the following composition: 60 μg biotin, 400 μg folic acid, and 5.0 μg vitamin B_{12}.

The final concentration of a parenteral nutrition solution prepared from a 20% commercial solution of lipids (Lipofundin^R MCT/LCT, Braun Melsungen AG) was used as a reference point. This fat emulsion has the following composition: 100 g/L soybean oil, 100 g/L half chain triacylglycerols, 12 g/L lecithin from egg yolk and 25 g/L glycerol. It was assumed that 500 mL of this lipids emulsion is delivered with 1.5 L of parenteral nutrition solution.

During the photochemical treatment, the solutions were irradiated with a 150-W slide projector lamp in a 1-cm light path cuvette thermostated at 25°C. The solutions to be irradiated were prepared by mixing one volume of the lipid emulsion, one volume of 0.05 M phosphate buffer, pH 7.0, and one volume of the sensitizer (1.5×10^{-5} M) in the same buffer. For the experiments with the MVI-1 infusate, a volume of 10 μL of this infusate was first diluted to 1.0 mL with buffer.

Oxygen consumption during irradiation was followed by means of a Yellow Springs Instruments model 5300 biological oxygen monitor. The absorption spectra were recorded on a Rapid Scan Spectronic 3000 Diode Array spectrophotometer (Milton Roy, Ivyland, PA, USA). The concentration of thiobarbituric acid reactive substances (TBARS)[7] in the samples was determined immediately after irradiation according to the method of Ohkawa et al.[8]

The frontier molecular orbital (FMO) calculations and full geometry optimizations for RF, FMN, and FAD were performed by using the semiempirical PM3 method[9] implemented in the Spartan package.[10] Our analysis also makes use of other important treatments that can assess compound reactivity like the soft-hard acid-base theory as proposed by Pearson[11,12] and recently established on solid and almost quantitative grounds by means of Density Functional Theory (DFT).[13–17] For a molecular system made out of electrons and nuclei reaching an equalibrium state, DFT shows that the electronegativity χ and the absolute hardness η are the first and the second derivatives of the electronic energy with respect of the number of electrons, respectively. Namely

$$\chi = -\left(\frac{dE}{dN}\right)_v \text{ and } \eta = \frac{1}{2}\left(\frac{d^2E}{dN^2}\right)_v \quad (1)$$

where v is the external potential due to the nuclei. Moreover, the electronegativity is not a simple function of the state of the system. Instead, it depends if the system can only loose electrons (χ = I, the ionization potential) or if it can only gain electrons (χ = A, the electron affinity). It turns out that when we deal with molecular systems for which both gaining and loosing of electrons are allowed, finite approximations are used.[13–15] Thus, Eq. 1 becomes

$$\chi = \frac{I+A}{2} \text{ and } \eta = \frac{I-A}{2} \quad (2)$$

We used these concepts in a suitable manner to predict the chemical behavior of RF, FMN, and FAD against oxygen uptake and the feasibility of photo-oxidation processes where the various electronic and steric effects of the substituents and heteroatoms make predictions difficult.

The manner to learn about preferential site properties within the molecule is to draw the corresponding molecular surface for a specific property after the self-consistent field (SCF) procedure. Actually, the full molecular electrostatic potential is very useful for this because, as it has been shown recently,[18] potential reactivity not only to incoming electrophiles but also to nucleophiles can be well described by plotting its surface. The electrostatic potential $\phi(r)$ that the electrons and nuclei of a molecule create at each point r in the surrounding space is given by

$$\phi(r) = \sum_A \frac{Z_A}{|\mathbf{R}_A - r|} - \int \frac{\rho(\mathbf{r}')dr'}{|r' - r|} \quad (3)$$

where Z_A is the charge on nucleus A located at R_A and $\rho(r)$ is the full electron density of the molecule written in terms of the electron occupation number n_i for the molecular orbital ψ_i (r),

$$\rho(r) = \sum_{i=1}^{OCC} n_i|\psi_i(r)|^2 \quad (4)$$

where the sum runs over the occupied molecular orbitals. It is noteworthy that $\phi(\mathbf{r})$ is a real physical property that can be determined computationally or experimentally by diffraction methods. Moreover, any other molecular reactivity index derived from it will correspond also to experimentally useful parameters.

Results

After irradiation of a commercial lipid emulsion in the presence of RF and FMN with polychromatic visible light, molecular oxygen consumption is observed, which in turn, increases when the fraction of energy absorbed by the sensitizer is increased (*Figure 1*). The fractions of absorbed energy at 450 nm (one minus the transmittance, 1-T) shown in *Figure 1* were obtained by using the following concentrations of the sensitizers: 5.0×10^{-6} M, 1.0×10^{-5} M, 1.5×10^{-5} M, 2.0×10^{-5} M, and 2.5×10^{-5} M. The concentrations of the lipid emulsion and of the flavins (5.0×10^{-6} M) used in this work, are similar to those in a traditional parenteral nutrition infusate. As seen in *Figure 1*, RF is more effective than FMN as sensitizer; whereas FAD, within the same concentration range, shows little or no effect as a sensitizer.

In *Figure 2*, the oxygen consumption of solutions of: 5.0×10^{-6} M FMN, the lipid emulsion and 1.0×10^{-3} M ascorbic acid (FMN/Lip/Vit. C); MVI-1 solution (containing 5.0×10^{-6} M FMN and 1.0×10^{-3} M ascorbic acid) and the lipid emulsion (MVI/Lip.); 5.0×10^{-6} M FMN and 1.0×10^{-3} M ascorbic acid (FMN/Vit. C) irradiated with polychromatic visible light is shown. *Figure 3* shows the photodecomposition that occurs in a solution of vitamin C (1.0×10^{-3} M) irradiated with polychromatic light in the presence of FMN (5.0×10^{-6} M). The absorption bands of flavin do not interfere with those of vitamin C mainly because of the high concentration of the ascorbic acid.

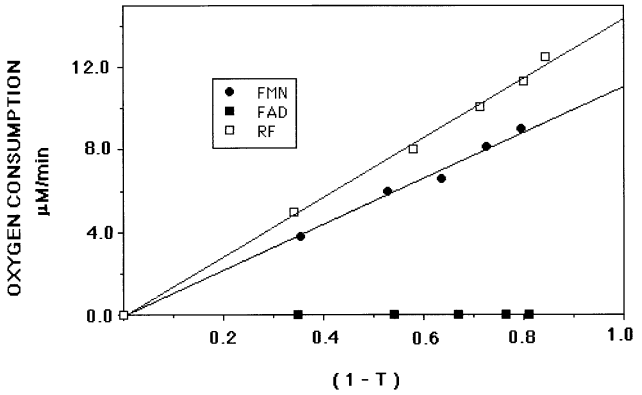


Figure 1 Oxygen consumption rate during the irradiation of a commercially lipid emulsion in the presence of different concentrations of flavins (5.0×10^{-6} M, 1.0×10^{-5} M, 1.5×10^{-5} M, 2.0×10^{-5} M and 2.5×10^{-5} M) expressed as the fraction of the energy absorbed (1-T) by the sensitizers. The transmittance (T) was measured at 450 nm.

The formation of TBARS during this process was also determined. These experiments used a concentration of 2.5×10^{-5} M of flavin because the concentration of TBARS formed was relatively low. The results are shown in *Figure 4*. The net effect observed for RF is greater than that for FMN. The antioxidative effect of vitamin C (1.0×10^{-3} M) is also apparent in this assay.

The results of molecular orbital calculations for RF, FMN and FAD are given in *Table 1*, where theoretical results for the solvated species $RF \cdot 9H_2O$ built by incorporating a H_2O molecule to each site of RF having the capacity to form hydrogen bonding with the solvent are also included. In general, the ionization energy and the electron affinity for RF show minor differences with respect to the solvated species; therefore, the same trend is found for their molecular electronegativities; however, the dipole moment is drastically reduced for the solvated species. *Figure 5* shows the electrostatic potential isosurface for RF, FMN, and FAD.

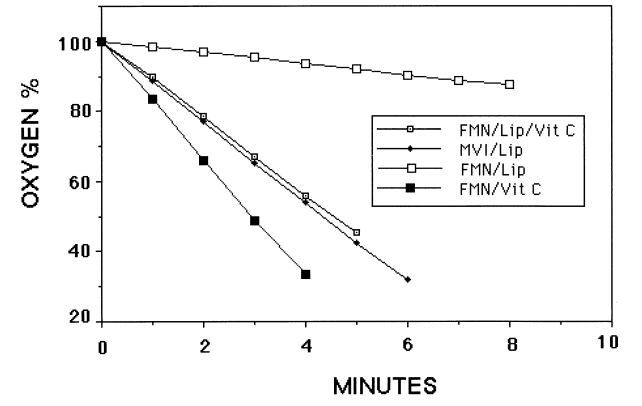


Figure 2 Oxygen consumption during the irradiation with visible light of solutions composed by: 5.0×10^{-6} M FMN, the lipid emulsion and 1.0×10^{-3} M ascorbic acid (FMN/Lip/Vit. C); MVI-1 solution (containing 5.0×10^{-6} M FMN and 1.0×10^{-3} M ascorbic acid) and the lipid emulsion (MVI/Lip.); 5.0×10^{-6} M FMN and the lipid emulsion (FMN/Lip.); and 5.0×10^{-6} M FMN and 1.0×10^{-3} M ascorbic acid (FMN/Vit. C).

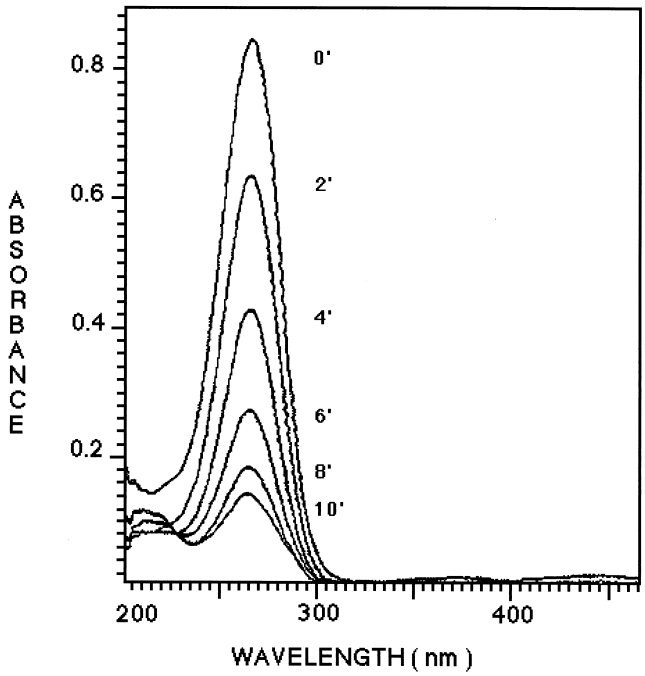


Figure 3 Absorption spectra of 1.0×10^{-3} M ascorbic acid solutions irradiated for 0, 2, 4, 6, 8, and 10 min with visible light in the presence of 5.0×10^{-6} M FMN, in 0.05 M phosphate buffer, pH 7.0. The solutions were diluted 10 times before measurements.

Discussion

The molecular oxygen consumption observed during the irradiation of a lipid emulsion in the presence of RF or FMN indicates the occurrence of a peroxidative reaction. The excited flavin molecule has a redox potential sufficiently negative to accomplish the initial hydrogen atom abstraction.[3] Furthermore, the very efficient intersystem crossing ($\phi_{isc} = 0.7$) in fluid solution at room temperature[6] and the high lifetime of flavins phosphorescence[19] suggest that the flavins triplet state is the most probable species to begin this process. This is depicted in the following scheme, where

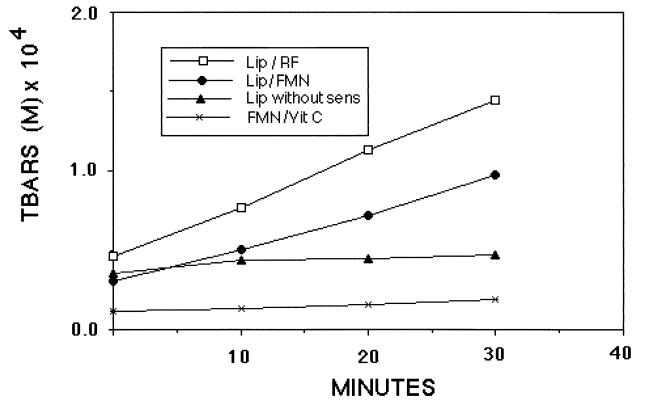


Figure 4 TBARS production during the irradiation with visible light of solutions composed by: the lipid emulsion and 2.5×10^{-5} M RF (Lip/RF); the lipid emulsion and 2.5×10^{-5} M FMN (Lip/FMN); the lipid emulsion (Lip without sens.) and 2.5×10^{-5} M FMN with 1.0×10^{-3} M ascorbic acid (FMN/Vit. C).

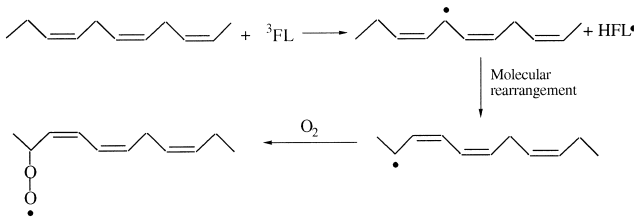
Table 1 Molecular electronic properties for RF, FMN, and FAD

Property	RF	RF.9H_2O	FMN	FAD
Molecular charge	0	0	− 1	− 2
Heat of formation ΔH_f,kcal/mol	−211.996	−730.478	−453.420	−697.353
Ionization potential, eV	8.116†	8.156*	4.746†	4.111
Electron affinity, eV	2.504†	2.843*	0.252†	−0.588†
Molecular electronegativity, eV	5.310	5.599	2.499	1.762
Dipole moment μ, Debye	7.773	3.550	12.720	26.577

*From PM3 methods.
†Calculated from total energies by using DFT methods.

^{3}FL represents a flavin in triplet state and HFL · is a flavosemiquinone free radical.

The formed peroxy radical can abstract H · from another fatty acid causing an autocatalytic chain reaction. HFL · could have the same function or can suffer the following molecular rearrangement:



$$HFL\cdot + HFL\cdot \rightarrow H_2FL + FL$$

$$H_2FL + O_2 \rightarrow FL + H_2O_2$$

where H_2FL represents the fully reduced flavin. From these reaction schemes it is evident that the H_2O_2 generated can also play an oxidative role. The characteristics of H_2O_2 production in a parenteral amino acid solution modeled on a commercially available paediatric parenteral amino acid solution have been examined previously.[20] In a recent paper[21] it has also been demonstrated that the multivitamin preparation was the major contributor to the peroxides generation.

The molecular orbitals obtained for RF using the semiempirical PM3 method, show that the quinone condensed aromatic rings drive both the HOMO and LUMO levels. In contrast, for FMN (and also for FAD), the HOMO is located entirely on the phosphate group and its LUMO is driven again by the aromatic quinone rings. In addition, for FAD, the sugar moiety does not play an important role in the frontier orbital description. Thus, whereas for RF, the frontier excitation process involves the π-orbitals of the aromatic groups exclusively, this situation does not hold true for either FMN or FAD. For both FMN and FAD, the electron must travel from one end of the molecule (phosphate group) to the aromatic ring region.

The capacity of a molecule to withdraw and accept negative charge is clearly related to its electronegativity χ. *Table 1* shows the calculated values of χ for RF, FMN, and FAD, and it is clear that χ(RF) >χ(FMN) ⩾ χ(FAD). Thus, riboflavin is more likely to undergo a reduction process than either FMN or FAD; it is also more efficient in this process and the results discussed above agree with this behavior.

The greater efficiency of RF with respect to FMN as sensitizer and the null sensitizing effect of FAD in the lipid emulsion exposed to the visible light, can be interpreted on the basis of their distribution in the organic and aqueous phases. In micellar systems, simpler than the lipid emulsion of a parenteral nutrient, it has been found that the percentage of RF incorporated into micelles in aqueous solution is quite large.[22] The ionic character of FMN limits its incorporation in to the lipid phase, and FAD, and even more ionic species, would remain basically in the aqueous phase and therefore not exert a peroxidative effect.

Figure 5 shows the geometry attained by each sensitizer

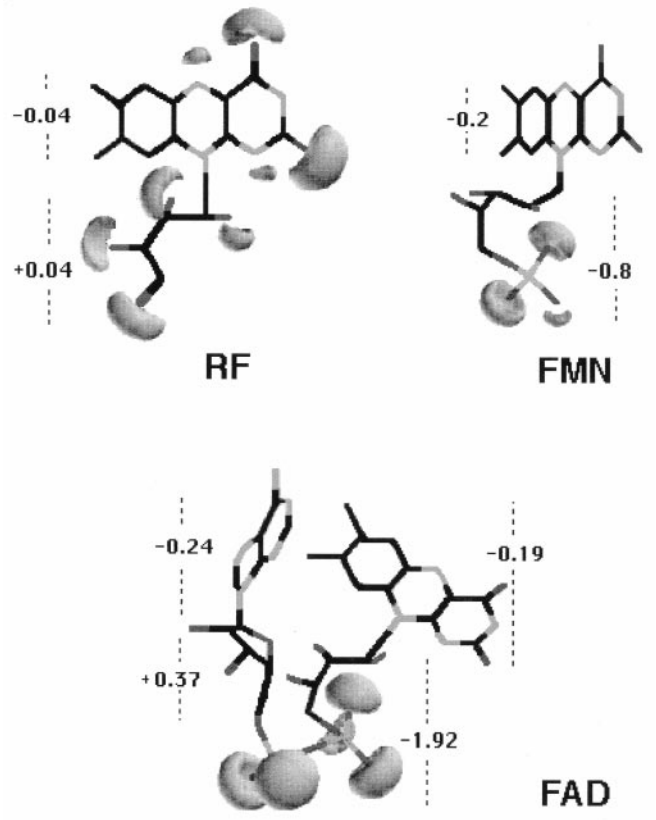


Figure 5 Electrostatic potential isosurface for: RF at −50 kcal/mol; FMN at −196 kcal/mol; and FAD at −200 kcal/mol. Regional charges interpolated from the SCF electrostatic potential are also indicated.

after selfconsistency. RF is characterized by the ribityl side-chain pointing outward the molecular plane formed by the aromatic groups, its electrostatic map at the −50 kcal/mol isosurface value covers practically all the main heteroatoms of the molecule.

Therefore, there is no preferential interaction against (+) ions. There are no large differences among −OH groups of the ribityl chain when compared with −C = O groups from the aromatic ring. Besides, N atoms from the isoalloxazinic ring are really less active against (+) charged species and overall, the molecule shows little charge separation among the molecular regions. However, the situation is different for FMN and FAD.

Figure 5 also shows that the electrostatic potential reaches large negative values (−196 kcal/mol for FMN and −200 kcal/mol for FAD), so there is a considerable attraction for positive ion species around the phosphate groups exclusively, leaving the organic molecular region practically free from intermolecular interactions at that level of interactions. Moreover, the final geometry for both molecules show large steric hindrance around the phosphate groups; for FAD, the organic aromatic groups from both the flavin and the sugar moieties reach one end of the molecule with a large volume far from the phosphate groups. Charges over the different molecular fragments for each sensitizer are also shown in *Figure 5* and it is clear that FAD is a very polar molecule. All of this, makes it difficult for it to be incorporated into the organic phase. The ionicity, is almost completely located at the phosphate region giving rise to a large dipole moment. For FMN, its dipole moment reaches a value between those of RF and FAD but closer to RF. Thus, whereas RF has the capacity to move to the organic phase, FMN and FAD do not. In addition to this, FAD also achieves a geometry with the large organic groups at one end of the molecule, thus creating a steric hindrance for its entrance to the organic micellar phase. After studying the sensitizing effect of the MVI, normally added to the lipid emulsion of the parenteral nutrients, it was found that molecular oxygen consumption was considerably greater than that observed in the presence of FMN (at the same concentration of this compound in MVI-1). This greater molecular oxygen consumption is attributable to the antioxidative effect of vitamin C present in the MVI. The oxygen consumption of the lipid emulsion when irradiated in the presence of MVI, is similar to that observed in the presence of vitamin C and FMN. This oxygen consumptions is essentially attributable to the photoconversion of the vitamin C sensitized by FMN. It is also possible that the vitamin E present in the multivitamin infusate could act as an antioxidant. However, it is important to underline that the ascorbic acid concentration in the infusate is 23 times higher than that of vitamin E and that vitamin C can reduce the α-tocopheryl radical (produced during the antioxidant action of vitamin E) back to α-tocopherol.

When measuring the concentration of TBARS formed as a consequence of the photoinduced lipid peroxidation it was found again that RF is more effective than FMN as a sensitizer. This experiment shows that vitamin C plays a very effective antioxidative role. In spite of the fact that vitamin C present in the parenteral nutrient guarantees the stability of the lipid emulsion exposed to the action of the visible light, it is necessary to consider that an important part of this vitamin could be photochemically destroyed and consequently the real dose that the patient would be receiving would be substantially smaller. It is also important to emphasize that the oxidation products of vitamin C have been described as agents that induce glycation (nonenzymatic glycosylation),[23,24] because they can react with the amino groups of the amino acid and proteins through a Maillard reaction.[25,26] The finding that FMN can be responsible for the photochemical decomposition of amino acids,[27–30] lipids, and/or vitamin C make it advisable that these nutrients as well as compounded nutrient infusates be protected against the action of light. There are publications that mention the possible effect of visible light to explain alterations in hepatic functions associated with parenteral nutrition.[31–33]

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