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SCAVENGING ACTIVITY OF AZELAIC ACID ON HYDROXYL RADICALS *"IN VITRO"*

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Azelaic acid is an aliphatic dicarboxylic acid (HOOC-$(CH_2)_7$-COOH) which has recently been shown to have some practical therapeutic applications in skin diseases of different etiologies. It possesses diverse biological activities and its mechanisms of action are still under investigation. Azelaic acid, as disodium salt ($C_9$2Na), at concentrations from 0.05 mM to 1.0 mM is capable of inhibiting significantly the hydroxylation of l-tyrosine to l-DOPA due to hydroxylradicals ($HO^{\cdot}$) produced by Fenton reaction. Similarly $C_9$2Na significantly inhibits the heterogeneous photocatalytic oxidation of toluene to cresols, and the peroxidation of arachidonic acid (C20:4,n6), due to $HO^{\cdot}$ formed by dissolved oxygen in the presence of UV-irradiated semiconductor TiO_2 (photo-Fenton type reaction). $C_9$2Na decomposition and its by-products formation are quantifiable only at high $HO^{\cdot}$ concentrations. On the contrary, $C_9$2Na is not a scavenger of $O_2^{\cdot-}$ generated by xanthine-xanthine oxidase system. Under the same experimental conditions, mannitol behaves like $C_9$2Na. These data indicate that $HO^{\cdot}$ scavenging capacity of $C_9$2Na *in vitro*, and represent a useful tool for further investigations on the mechanisms of action of azelaic acid in biological systems.

KEY WORDS: Azelaic acid, Fenton and photo-Fenton type reactions, hydroxylradicals, mannitol

Azelaic acid (HOOC-$(CH_2)_7$-COOH) is a medium chain length saturated dicarboxylic acid which naturally occurs in rancid oleic acid, and, in man, in the urine of patients with ketosis, or with a congenital or acquired inability to beta-oxidase monocarboxylic acids.[1] Following administration by different routes, it is predominantly eliminated in the urine, but partly metabolized via beta-oxidation, and partly decarboxylated.[2] Azelaic acid is devoid of toxicity,[3] teratogenicity[3] and mutagenicity.[4]

Recognition over the past ten years of the biological activities and therapeutic potential of azelaic acid stems from pioneer studies on skin surface lipids and the pathogenesis of hypochromia in Pityriasis versicolor. In culture of the fungus Pityrosporum to which unsaturated fatty acids with double bonds in the 6–12 positions were added, dicarboxylic acids of chain length C_6 to C_{12} were formed; these were shown *in vitro* to be competitive inhibitors of tyrosinase (a key enzyme for melanogenesis) with an efficiency proportional to chain length.[5] A cream containing azelaic acid (C_9) proved capable of reverting to normal hyperipigmentary disorders related to increased activity of melanocytes, such as melasma, or their abnormal proliferation (lentigo maligna, i.e. melanoma in situ).[6] A significant biological activity was also observed on primary cutaneous malignant melanoma following oral and topical administration,[7] and *in vitro* experiments have shown that it affects proliferation and

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viability of murine and human melanoma cells and tumoral cells of other lines.[8] The antitumoral effect is associated with reversible inhibition of mitochondrial[9] and microsomal oxido-reductase,[10] and inhibition of DNA synthesis.[11,12] Among other properties, azelaic acid also exhibits antimicrobial activity,[13] which accounts for its effectiveness in the therapy of acne vulgaris.[14]

Its biological activities, therefore, are multiple and diverse, and its mechanisms of action deserve further investigations.

Since in the last years it has been suggested that reactive oxygen species (ROS) may play a pathogenic role on skin diseases positively affected by azelaic acid,[15,16] it seemed interesting to examine whether azelaic acid has an effect on the *in vitro* generation of ROS, in particular superoxide anion radical ($O_2^{\cdot-}$) and hydroxyl radical ($HO^{\cdot}$).

MATERIALS AND METHODS

Azelaic acid (99% pure) was purchased from Fluka and was salified with NaOH (Merck AG) ($C_9$2Na).

$O_2^{\cdot-}$ Generation by Xanthine-xanthine Oxidase System

40 μg Xanthine (Sigma Chem. Co., St. Louis, MO., USA), 1 mM EDTA and 0.02 mM ferricytochrome c (type III, Sigma) were diluted in 2 ml of PBS (pH 7.4). Then 0.2 ml of 0.2 U/ml dialyzed xanthine oxidase (Sigma) was added in the absence and presence of 10^{-5}–10^{-3} M disodium azelate ($C_9$2Na). The initial linear rate of cytochrome c reduction was followed spectrophotometrically at 550 nm and 37°C in the Acta VI recording spectrophotometer equipped with a circulating water bath, using an extinction coefficient for cytochrome c (reduced-oxidized) of 21 mM cm^{-1}.[17]

$HO^{\cdot}$ Generation by Fenton Reaction

In the classical Fenton reaction, ferrous ions (Fe^{++}) react with hydrogen peroxide (H_2O_2) giving rise to powerful hydroxyl radicals ($HO^{\cdot}$). Hydroxy radicals are capable of inducing: A) the hydroxylation of l-tyrosine to l-dihydroxyphenylalanine (l-DOPA).[18] The spontaneous l-DOPA autooxidation at physiological pH can be prevented by the presence of ascorbic acid in the reaction medium. In our experiments we have used 200 μg of l-tyrosine, 30 μg $FeSO_4$, 20 μg ascorbic acid and 30 μg 5% H_2O_2, all dissolved in 2 ml 0.02 M PBS at pH 7.2, in the absence and presence of 10^{-5}–10^{3} M disodium azelate. L-DOPA and dehydroascorbic acid formation, l-tyrosine and ascorbic acid consumptions have been evaluated by reversed-phase high performance liquid chromatography (RP-HPLC). Condition: column: Supelcosil LC-18 (25 cm × 4.6 mm, 5 μm packing) plus w/Supleguard guard column; mobile phase: acetonitrile; 0.02 M K_2HPO_4, 1 mM heptane sulfonic acid (pH to 3.0 with H_3PO_4), 5:95. Column temperature 30°C; flow rate: 1 ml/min; Detector UV at 280 nm. B) The peroxidation of Arachidonic acid. 100 μg of arachidonic acid were added to 2 ml of 0.02 M PBS at pH 7.2 containing 30 μg $FeSO_4$ and 30 μl 5% H_2O_2 in the absence and presence of 0.1–10 mg disodium azelate. After 30 min incubation at room temperature under agitation with magnetic stirrer, the solutions were acidified to pH 2–3 with diluted HCl. Arachidonic acid was extracted by peroxide free diethyl ether and analysed by the use of capillary direct gas chromatography-mass

spectrometry (GC-MS). A Hewlett and Packard 5890 gas chromatograph was configured with a capillary split-splitless inlet and as HP 5970 mass selective detector (MSD) equipped with capillary direct interface. Instrument control and data reduction were accomplished with the HP 59970 MS Chem Station. Conditon; column FFA-P (50 m × 0.32 mm × 0.52 μm, Hewlett and Packard); injection (1 μl), splitless; oven temperature: 50°C to 180°C at 10°C/min, then to 240 °C at 2°C/min and hold; transfer line temperature: 220°C; carrier gas, helium (1 ml/min). Scan data were acquired from 50 to 550 amu at scan rate of 1.71 scans/s and an electron mutliplied voltage of 2000 emv. Arachidonic acid was quantified by comparison with peak area of reference standard from Sigma Chem. Co. Peak area values given were the result of the integration of the total ion chromatograms.

HO˙ Generation by Photo-Fenton Type Reaction

It is known that the real oxidant in the aqueous UV irradiated TiO_2 system is considered to be the hydroxyl radical[16] as in Fenton's reagent. We have investigated the effects of $C_9$2Na on the heterogeneous photocatalytic oxidation of toluene in the presence of illuminated TiO_2 powder[19] (photo-Fenton type reaction). 200 mg of photocatalist (TiO_2, 99.99% Anatase from Aldrich) in a mixture of 5 ml of 0.02 M phosphate buffer, pH 7.2 and toluene (500 μl) was irradiated up to 4 h in the presence of 1.0–50 mg of $C_9$2Na, by a 500 W high pressure mercury arc lamp in a 5 ml Pyrex glass round bottomed flask with a condenser cooled with running water. The solution was saturated with oxygen in the air and was stirred by a magnetic stirred. After photolysis, the solution was made acidic by diluted HCl addition, then extracted three times under peroxide free diethyl ether, and finally concentrated under a reduced pressure (Mix A). Before extractions, 50 μg of butylated hydroxyanisole (BHA) were added as internal standard. The neoformed compounds from toluene (benzaldehyde, benzylalcohol, diphenyl, ortho-, meta- and para-cresols) present in Mix A, were analysed by GC-MS system. Conditions: column: Nukol (60 m × 0.25 mm × 0.25 μm, Supelco); injection (1 μl): splitless; oven temperature: 50°C to 180°C at 10°C/min and hold; transfer line temperature: 180°C; carrier gas helium (0.5 ml/min). Scan data were acquired from 50 to 500 amu at scan rate of 1.71 scans/s and a electron multiplier voltage of 2000 emv. Benzahaldehyde, benzylalcohol, diphenyl, ortho-, meta- and para-cresols were quantified with peak areas of reference standards from Sigma Chem, Co. The amount of a gaseous product, CO_2, was determined according to Kraeutler e Bard.[20] Degradation of $C_9$2Na and identification of some by-products of its oxidation (Mix A) were evaluated by GC-MS on FFA-P column as described above.

MANNITOL

In some experiments the well known hydroxyl radical scavenger mannitol (MAN) (Merck AG)[21] was used at the same concentrations as $C_9$2Na and its effects were compared with those obtained by $C_9$2Na.

STATISTICAL ANALYSIS

Statistical significance was determined according to Student's t-test.

RESULTS

$C_9$2Na is not a scavenger of $O_2^{\cdot-}$ generated by the xanthine-xanthine oxidase system at concentrations from 10^{-5} M to 5×10^{-3} M. On the other hand, Figures 1 and 2 show that $C_9$2Na is capable of inhibiting the hydroxylation of l-tyrosine to l-DOPA induced by $HO^{\cdot}$ generated by the Fenton reaction. The % inhibition is greater at 5 min, but stabilizes after 10 min incubation. Mannitol (MAN), at the same concentrations as $C_9$2Na, produces similar effects (data not shown).

The inhibition of $C_9$2Na or MAN on arachidonic acid peroxidation induced by $HO^{\cdot}$ is also clear, but much less striking as compared with inhibition of l-DOPA formation and, moreover, is observed at higher concentrations. After 30 min incubation with the Fenton system, 10 mg of $C_9$2Na and 10 mg of MAN are able to inhibit 41% and 35% peroxidation of 100 μg C20:4 respectively.

Also in the photo-Fenton type reaction (see Materials and Methods) $C_9$2Na is capable of inhibiting significantly the formation of ortho, meta and para cresols and, to a lower extent, of benzaldehyde and benzylalcohol, starting from toluene as substrate (Figure 3). In Table I the % inhibition achieved by $C_9$2Na and MAN at different concentrations after 4 h of UV irradiation is reported. At shorter irradiation time the % inhibitions by $C_9$2Na or MAN are higher than after 4 h (data not reported), and in all cases, always dose dependent (Table 1).

It is worth mentioning that during the photocatalytic oxidation of toluene, $C_9$2Na is partly degraded and that its consumption is directly correlated to the period of UV irradiation (Figure 4). Several by-products are formed by the oxidation of the diacid and further investigation will be necessary for their complete identification. However,

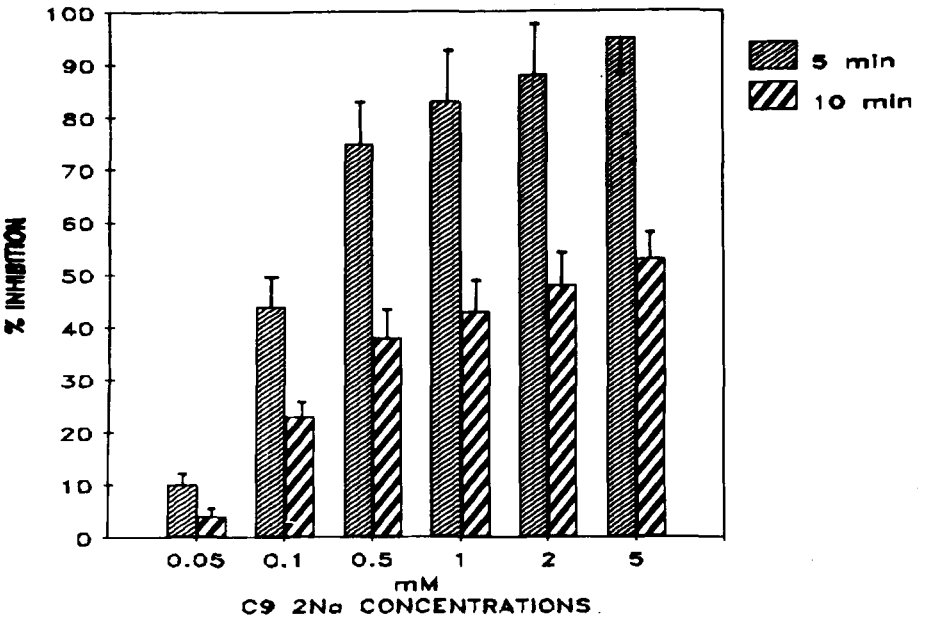

FIGURE 1 Percent inhibition, at 5 and 10 min. of incubation, of hydroxylation of l-tyrosine to l-DOPA induced by $HO^{\cdot}$ radicals (Fenton reaction) in the presence of different $C_9$2Na concentrations (in mM). Each result represents the average of 5 experiments $\pm$ SD. After 10 min of incubation the percent inhibition remains constant. Under the same experimental conditions, MAN behaves like $C_9$2Na.

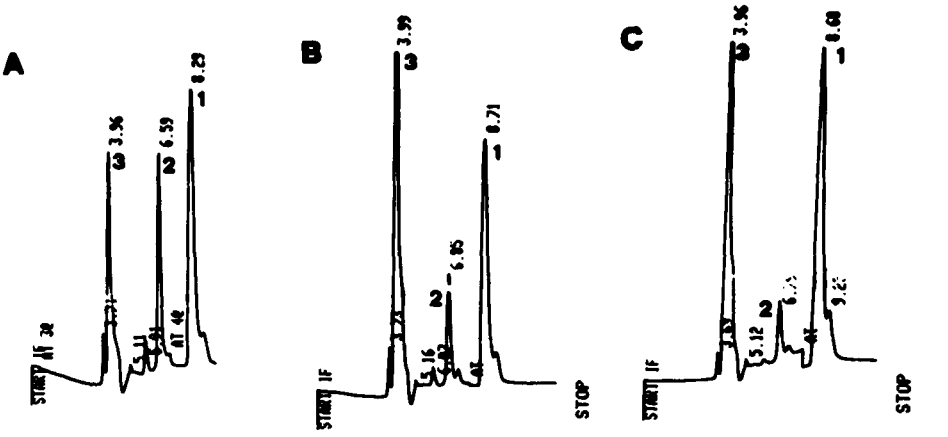

FIGURE 2 HPLC separation of l-tyrosine, l-DOPA and Vitamin C on RP18 column. A: l-DOPA produced during Fenton reacition after 10 min incubation. 1; l-tyrosine; 2: l-DOPA; 3: Vit. C; B: the same but in the presence of 10^{-4} M $C_9$2Na; C: the same but in the presence of 10^{-3} M $C_9$2Na. The reduction of l-DOPA production is parallel to a decreased oxidation of l-tyrosine. Under the same experimental condition MAN behaves like $C_9$2Na.

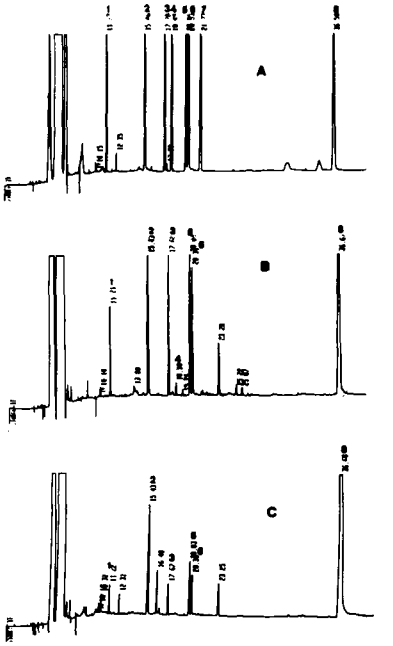

FIGURE 3 Gas-chromatographic separation of products generated following photo-Fenton type reaction on toluene. A: separation of standards: 1; benzaldehyde; 2: benzylalcohol; 3: ortho-cresol; 4: diphenyl; 5: meta-cresol; 6: para-cresol; 7: dibenzyl; 8: parahydroxyanisol (internal standard). B: separation of aromatic products generated following photo-Fenton type reaction (500 μl toluene, 5 ml phosphate buffer pH 7.2 C: the same but in the presence of 50 mg $C_9$2Na. Under the same experimental conditions MAN behaves like $C_9$2Na.

TABLE I

Effect of different concentrations of C9 2Na and MAN on yields for photo-Fenton type reaction of Toluene (500 μl). Time of UV irradiation: 4 h. PhCHO: benzaldehyde; BzOH: benzylalcohol; o^-, m^-, p-Cr: ortho-, meta- and para-cresols; Diph: diaphenyl. Each result represents the average ± SD of 5 determinations.

C9 2Na (mg)	PhCHO	PRODUCTS (μmol) BzOH	o^- Cr	Diph	m-Cr	p-Cr	Total μmol	% inhibition
a	17.4 ± 2.7	33.6 ± 3.6	24.7 ± 3.9	2.2 ± 0.4	25.5 ± 3.1	20.6 ± 3.8	124.0	-
1	17.0 ± 3.0	31.8 ± 4.2	23.5 ± 3.6	2.3 ± 0.3	25.1 ± 2.8	21.2 ± 3.3	120.9	2.5
5	16.7 ± 2.4	29.6 ± 4.5	21.9 ± 4.0	1.9 ± 0.4	22.7 ± 3.3	19.2 ± 2.7	112.0	9.7
10	15.8 ± 2.5	25.8 ± 4.5*	19.7 ± 3.6*	1.3 ± 0.4*	20.6 ± 3.3*	17.5 ± 3.1*	100.7	18.8
25	11.4 ± 3.1**	21.2 ± 3.6**	12.8 ± 2.4**	-	13.9 ± 2.2**	11.3 ± 2.1**	70.6	43.1
50	5.1 ± 1.5**	15.4 ± 2.7**	4.9 ± 1.6**	-	6.1 ± 1.4**	5.2 ± 1.6**	36.7	70.4
MAN (mg)								
10	14.5 ± 2.6*	24.2 ± 3.6*	17.6 ± 3.1*	-	18.2 ± 3.8*	15.3 ± 3.3*	89.8	27.6
50	3.01 ± 1.3**	11.8 ± 1.8**	2.5 ± 1.2**	-	3.6 ± 1.6**	3.2 ± 1.4**	24.1	80.6

*$p < 0.01$ tested against values of control (a)
**$p < 0.001$ tested against values of control (a).

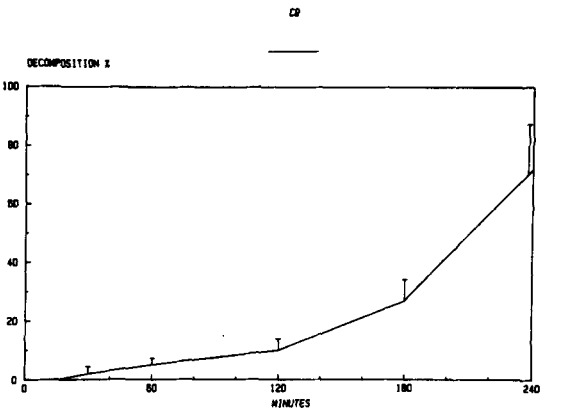

FIGURE 4 Time course of decomposition (%) of $C_9$2Na during the photo-Fenton type reaction. Each result represents the average of 5 determinats ± SD.

one of the first by-products identified by GC-MS on FFA-P capillary column (see Materials and Methods) is represented by the 7-oxooctanoic acid. This is consistent with the radical nature of the oxidation of $C_9$2Na, which can act as a scavenger of HO·.

DISCUSSION

The present study demonstrates that disodium azelate ($C_9$2Na) is capable of inhibiting significantly the oxidation of aromatic compounds and the lipoperoxidation of arachidonic acid (C20:4,n6), which are due to hydroxyradicals (HO·) generated by Fenton and photo-Fenton type reactions (Figures 1–3, Table I). The inhibition of peroxidation of C20:4,n6 requires $C_9$2Na concentrations much higher than those necessary to inhibit oxidation of tyrosine and toluene. Undoubtedly it is much easier, and energetically more favourable, for hydroxy radicals to attack unstable substrates such as arachidonic acid than to act as hydroxylating agents on aromatic compounds.

In the Fenton reaction, H_2O_2 decomposes into hydroxyl radicals and hydroxide anions by reductive cleavage with ferrous ions as follow;

$$H_2O_2 + Fe^{2+} \rightarrow HO^{\cdot} + HO^{-} + Fe^{3+}.$$

It may be thought that the inhibitory effect on l-DOPA formation might be due to an aspecific chelating action of $C_9$2Na on ions since some polycarboxylic acids (such as EDTA), are known to chelate metals. Therefore, for a better understanding of the phenomenon, we have employed the heterogeneous photocatalytic reaction on a semiconductor material (TiO_2), that is a system for the production of HO· which does not require the presence of the metal ion catalysts. It was shown[19] that this reaction proceeds via the same mechanisms as the Fenton reaction and consequently it was named "the photo-Fenton type reaction". The real oxidant in the aqueous TiO_2 system is considered to be the HO·, which is generated from both the catodic reaction product, H_2O_2(19), as in a Fenton reaction:

$$hv \rightarrow e^{-} + h^{+}, 2h^{+} + H_2O \rightarrow 1/2O_2 + 2H^{+}$$

$$O_2 + 2H^+ + 2\,e^- \rightarrow H_2O_2$$

$$H_2O_2 + \begin{cases} O_2^- \rightarrow HO^{\cdot}\ HO^- + O_2 \\ h\,v \rightarrow 2HO^{\cdot} \\ e^-\ (TiO_2) \rightarrow HO^{\cdot} + HO^- \end{cases}$$

and from the anodic oxidation of HO^- or H_2O[19]:

$$H_2O\ (ads) + h^+ \rightarrow HO^{\cdot} + H^+$$

$$HO^- + \longrightarrow HO^{\cdot}$$

It is interesting to note that, among the products of toluene oxidation, $C_9$2Na inhibits the formation of ortho, meta and para-cresols more efficiently than that of benzaldehyde or benzylalcohol (Figure 3, Table I) Cresol formation and side chain oxidation (benzaldeyde, benzylalcohol) proceeed through two different mechanisms: cresols are synthetized via a hydroxycyclohexadienyl radical formed by $HO^{\cdot}$ addition to the aromatic ring, while benzaldehyde and benzylalcohol via a cation radical which is known to lose a proton irreversibly to give a benzyl radical. Therefore, it is possible that $C_9$2Na inhibits preferentially the compounds starting from hydroxycyclohexadienylradical with subsequent hydroxylation of the aromatic ring, rather than those deriving from cation radical with oxidation of side chain.

It is worth mentioning that during the photocatalytic oxidation of toluene, $C_9$2Na is partly degraded and that the rate of its consumption depends on the period of UV irradiation. One of the first products of $C_9$2Na oxidation is represented by 7-oxooctanoic acid, which suggests a radical mechanism of decomposition:

$$HO^{\cdot} + R\text{-}CH_2\text{-}COO^-\ Na^+ \longrightarrow R\text{-}CH_2\text{-}COO + HO^-$$

$$R = NA^+\ {}^-OOC\text{-}(CH_2)_6$$

$$R\text{-}\dot{C}H_2 + CO_2$$

7-oxooctanoic acid

It is possible that also the other carboxyl ion of $C_9$2Na can be affected simultaneously. Therefore a fairly complex sequence leading to several by-products does occur.

The proposed radical mechanism is consistent with the increased CO_2 formation during the experiment. In this connection, it is of interest that also saturated short chain monocarboxylic acids undergo decarboxylation to alkanes following the heterogenous photocatalytic reaction on TiO_2 powder.[20]

While being a scavenger of hydroxyradicals, azelaic acid is devoid of activity on superoxide radicals. In fact, at concentrations from 10^{-5} to 5×10^{-3} M, it does not affect the rate of reduction of cytochrome C induced by superoxide anion radical (O_2^-) generated by the xanthine-xanthine oxidase system.

From the foreging observations it seems likely that $C_9$2Na can be added to the list of well known radical scavengers such as thiourea, ethanol, dimethylsulphoxide, benzoate, mannitol, etc., also called radical interceptors,[21] which are not active towards superoxide anion radicals. In this context, it is important to underline that:
a) the effects of MAN on HO produced by Fenton or photo-Fenton type reactions

parallel those obtained by similar concentrations of $C_9$2Na (Table I); b) all these substances, including azelaic acid, though not specific, are oxidized by HO· at different rate, yielding new radicals. For example, HO· reacts with MAN or dimethysulphoxide $(CH_3)_2SO$) producing, respectively:[21]

$$HO\cdot + MAN \longrightarrow MAN\cdot + H_2O$$

$$HO\cdot + (CH_3)_2SO \longrightarrow CH_3S(OH)O + \dot{C}H_3.$$

In the case of azelaic acid, it must be pointed out that its decomposition only occurs in the presence of high HO· concentrations. The appearance of 7-oxooctanoic acid is detectable after 30 minutes of exposition to the Fenton-type reaction and its time course is complex, being autocatalytic (Figure 4). This suggests that, in addition to the HO· flux, other newly formed reactive species contribute to the diacid decompositon.

With lower fluxes of HO·, such as those generated in the classical Fenton reaction and in the peroxidation of arachidonic acid, notwithstanding the evidence that both 1-DOPA formation and arachidonic acid oxidation are inhibited (Figures 1,2), the decomposition of $C_9$2Na and the formation of by-prodcuts is not quantifiable by our analytical methods. In this case, the rate of decomposition may be so slow as to be kinetically insignificant.

These findings may be of interest with reference to the activity of azelaic acid in biological systems, where the HO· generation is minimal by comparison with our experiments *in vitro*, and therefore, the formation of radical by-products of $C_9$2Na is probably biologically irrelevant. Preliminary studies on cell cultures have shown that $C_9$2Na may act as a protective agent against the cytotoxic effects of UV irradiation or diphenol autoxidation in the culture medium.[22]

These data, showing the HO· scavenging capacity of $C_9$2Na *in vitro*, may represent a useful basis for further investigations into the mechanisms of action of azelaic acid in biological systems.

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