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The effect of folates on the oxidation reaction of methyl linoleate in micellar solutions

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The antiradical/antioxidant properties of folic acid (FA) and its conformational derivatives – 7,8-dihydrofolate (DHF), 5,6,7,8-tetrahydrofolate (THF) and 5-formyl-5,6,7,8-tetrahydrofolate (5-FTHF) – were studied at the physiological temperature and pH in the micellar aqueous system of the model reaction of methyl linoleate peroxidation with molecular oxygen. It was established that folates exhibit antioxidant properties, thereby inhibiting the peroxidation of methyl linoleate.

Quantitative kinetic studies of selected folates revealed the comparative growing order of the antiradical/antioxidant reactivities: FA < 5-FTHF < THF ≅ DHF.

Investigation of the antioxidant properties of folates is urgent for understanding their role in the prevention of pathologies in the body. They may have potential in treating numerous pathological conditions in which oxidative stress is a clinically important component.

Keywords: folate, methyl linoleate, peroxyl radical, micellar solution

INTRODUCTION

Folate is the generic term for the water-soluble B-complex vitamin B9, encompassing folic acid and its derivatives – dihydro-, tetrahydro- and formyl- compounds. Folic acid (N-[p-{(2-amino-4-hydroxy-6-pteridinyl methyl] amino}benzoyl]-L-glutamic acid) is the basic structural synthetic and the most oxidised form of folate.

Folic acid (FA) consists of three primary structures: a heterocyclic pterin (PT) moiety, *para*-aminobenzoic acid (*p*-ABA) and glutamic acid (Glu). The pterin core of the FA molecule consists of pyrimidine and pyrazine rings [1, 2].

Folic acid is *in vivo* converted through a series of enzymatic transformations. FA is reduced to 7,8-dihydrofolate (DHF), which is subsequently reduced to 5,6,7,8-tertahydrofolate (THF) and then enzymatically converted into 5-methyltetrahydrofolate (5-MTHF). 5-Formyltetrahydrofolic acid (5-FTHF), also known as folinic acid, is one of the coenzyme forms of FA that is produced commercially [2, 3] (Fig. 1).

Folic acid is important for many bodily functions, especially as it is required for the normal growth and division of cells. FA and its conformational structural forms are cofactors in the transfer of one-carbon groups to other metabolites and participate in reactions essential for many fundamental cellular functions [4, 5]. They are involved in the biochemical processes related to the biosynthesis of nucleic acids (DNA, RNA) and proteins, the repair of DNA, and the regulation of gene expression [6, 7].

Folic acid is an essential hematogenic agent and acts as coenzyme to regulate the generation of ferroheme [8]. Firstly, FA was identified as a factor of growth and antianemia. It is taken orally using various drug formulations [9], as well as, some supplements containing FA and Fe (II) complexes recommended as antianemia drugs. Folic acid forms water-insoluble complex compounds with divalent metals, such as Cu^{2+}, Fe^{2+} and Co^{2+},

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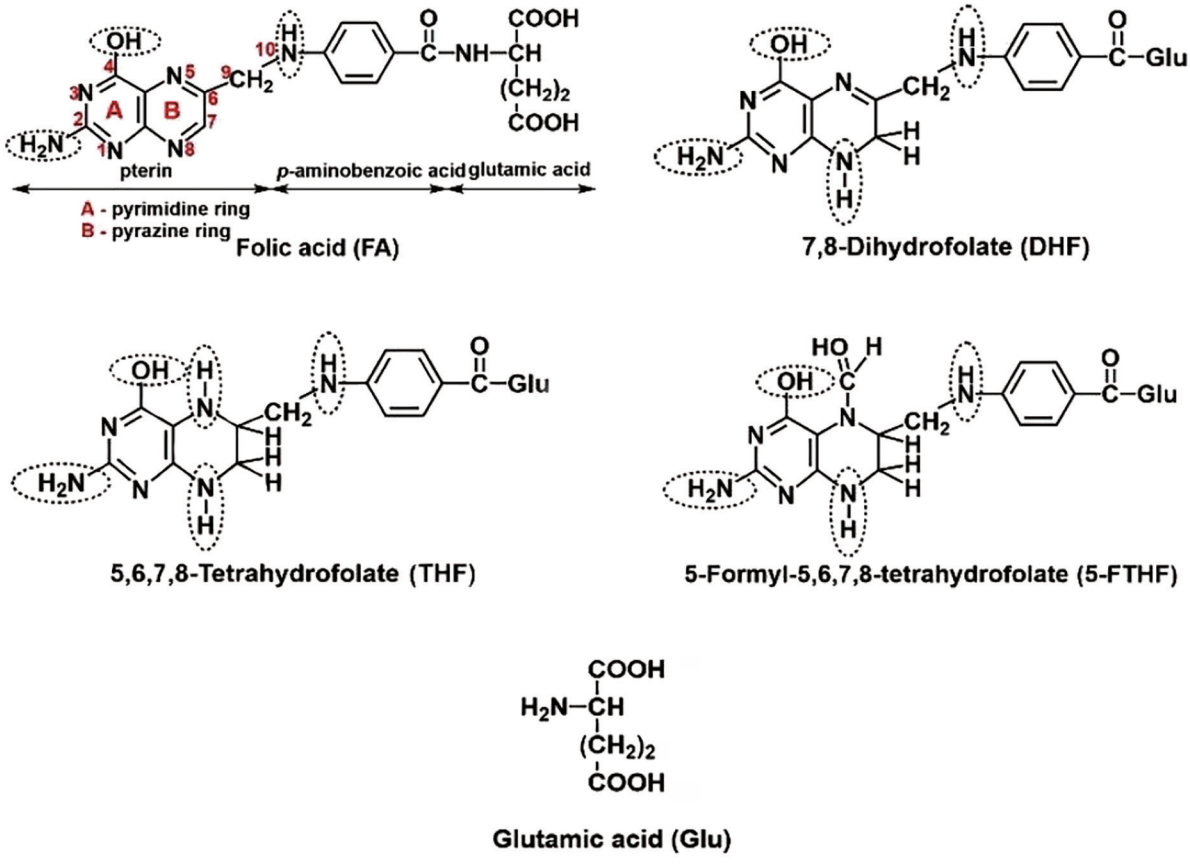


Fig. 1. Schematic molecular structures of folates and their possible antiradical/antioxidant sites. For folic acid, the main structural units are indicated

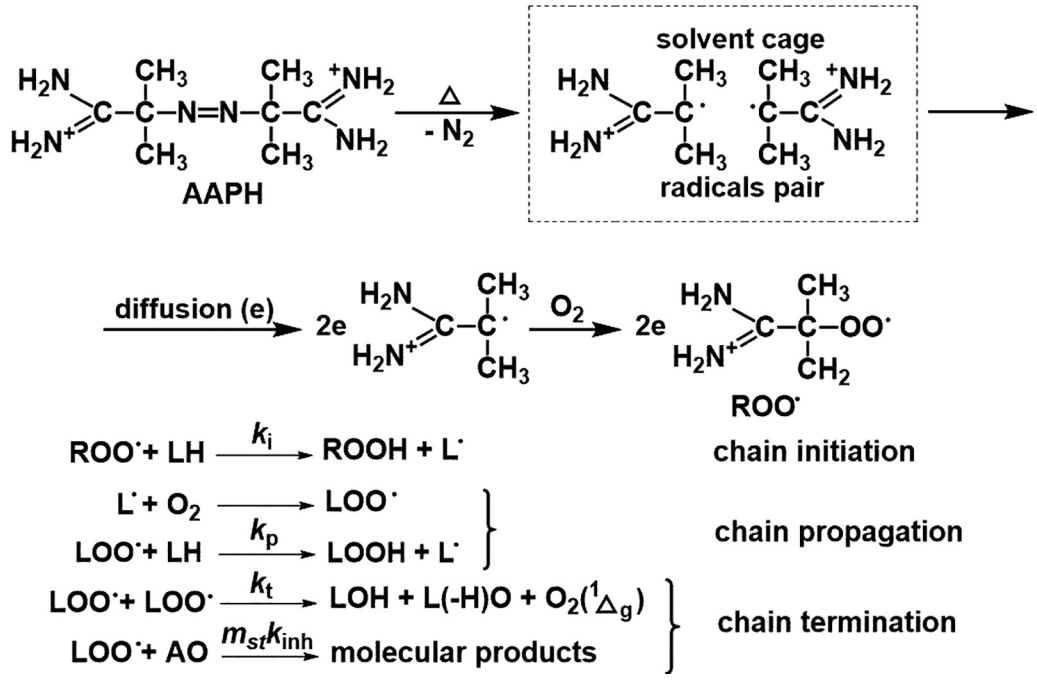
by coordination with its Glu or PT moieties. In biological fluids, specifically in blood, FA forms stable adducts with these cations [10–12].

Among the variety of mechanisms of bioactivities, the antioxidant and antiradical activities of folates are of special importance [4, 5, 13].

The chemistry of folates as antioxidants is particularly interesting due to their ability to protect cell lipids and DNA from oxidative damage. They can also protect the blood from oxidative destruction [14, 15]. They neutralise and 'scavenge' free radicals and other reactive oxygen-containing species (ROS), which can change the properties of biological membranes, attack other vital cell targets, affect the functional state of the cell, and lead to pathological conditions called 'oxidative stress' [16, 17].

The study of the antioxidant properties of folates in the oxidation reactions of methyl linoleate in micellar solutions is an urgent task. It is important to understand their effect on the model reaction of lipid peroxidation of cell membranes. The chain-free radical oxidation of methyl linoleate in micellar solutions, as a kinetic model of the biological process of lipid peroxidation, is used [17–19]. Through such systems, the reactivity of antioxidants is studied, the mechanism of action of which is mainly determined by their interaction with chain carriers – peroxyl radicals (Scheme). Peroxyl radicals are the predominant free radicals found in lipid oxidation in foods and biological systems under physiological conditions.

In this regard, to determine the antiradical and antioxidant properties of folates, the goal of this work was to investigate the ability of folic acid and its conformational derivatives to inhibit chain reactions of peroxidation in lipid model systems. Simultaneously, the aim was to reveal the comparative order of the antiradical/antioxidant activities of selected folates through kinetic measurements. The obtained results provide important information for identifying the chemical mechanisms of folate interaction with peroxyl radicals, which play an essential role in the process of oxidative stress in the body.



Scheme. Kinetic scheme of the chain oxidation of oxidative substrate by molecular oxygen in the presence of antioxidants. k_i and k_{inh} are the rate constants of initiation by peroxyl radicals and inhibition by antioxidants; k_p and k_t are the rate constants for the propagation and termination of the substrate, respectively, m_{st} is a stoichiometric factor

MATERIALS AND METHODS

Materials

Folic acid (FA), 7,8-dihydrofolate (DHF), 5,6,7,8-tetrahydrofolate (THF) and 5-formyl-5,6,7,8-tetrahydrofolate (5-FTHF), azoinitiator 2,2'-azobis(2-amidinopropane) hydrochloride (AAPH), methyl linoleate (ML) and Triton X-100 were purchased from the Sigma-Aldrich (USA) chemical company. The solvents, methanol (CH_3OH) and phosphate buffer (0.05 M, pH = 7.4) (NaH_2PO_4, Na_2HPO_4), were also purchased from the same company. In all experiments, deionised water with an electrical resistance of 18.2 Mom × cm at 25°C was used.

Kinetics of chain oxidation of methyl linoleate in micellar aqueous solutions

The chain-free radical oxidation of methyl linoleate with dioxygen was studied using an amperometric numerical complex (Oximeter YSI 5300 Biological Oxygen Monitor, USA).

The kinetics of methyl linoleate peroxidation in the presence of folates were studied by recording kinetic absorption curves of molecular oxygen during the reaction. The peroxidation reaction of methyl linoleate was initiated by the generation of peroxyl radicals through the thermal decomposition of water-soluble AAPH in the presence of dioxygen at a temperature of 37±0.1°C (Scheme). Trolox, a water-soluble analogue of α-tocopherol, was used as a standard.

A system consisting of methyl linoleate, Triton X-100/phosphate buffer and molecular oxygen was used as the micellar reaction system. The surface-active compound, Triton X-100, was used to obtain micellar systems, and methyl linoleate served as the oxidative substrate [20–22]. The Triton X-100/phosphate buffer solution was prepared using an ultrasonic device for a duration of 8 min. A mixture of Triton X-100/phosphate buffer (both 5×10^{-2} M) and methyl linoleate (2.5×10^{-2} M) was initially enriched with argon gas for 30 min, and then with oxygen gas for 5 min. After the mentioned processes, a solution of the thermal azo-initiator (AAPH) and a solution of the corresponding studied antioxidant (folates and trolox) were successively added to the reaction mixture. The final volume of the reaction system was 3 mL. During the enrichment and the measurement, the mixture was stirred in a thermostatically controlled reaction cell using a magnetic stirrer.

Analyses were carried out at a constant temperature of 37±0.1°C and at pH = 7.4, adjusted with a phosphate buffer. Concentrated solutions of DHF,

THF and 5-FTHF (2.5×10^{-4} M) were prepared in water, while the concentrated solution of FA (2.5×10^{-4} M) in methanol, and the concentrated solution of trolox (2.5×10^{-4} M) in ethanol. A concentrated solution of 7×10^{-2} M azo-initiator AAPH was prepared in a phosphate buffer.

RESULTS AND DISCUSSION

In Fig. 2, typical kinetic curves of oxygen absorption in the methyl linoleate peroxidation reaction are demonstrated at different concentrations of FA and trolox as a reference.

As follows from the data presented in Fig. 2a, upon addition of the antioxidant sample to the reaction mixture, there is a decrease in the average rate of oxygen uptake compared to the reaction in the absence of the antioxidant. This indicates that FA exhibits antioxidant properties.

At the same time, in the presence of the standard antioxidant trolox in the reaction system (Fig. 2b), a period of reaction induction is observed – the time after which the rate of oxygen absorption and peroxidation of methylinoleate significantly increases. The same effect is not observed in the presence of folic acid. This means that FA exhibits a weak antioxidant activity, despite the involvement of all reaction sites of the FA molecule (Fig. 1).

A similar effect as seen with FA is noted for its structural derivatives: DHF, THF and 5-FTHF. The corresponding kinetic curves are presented in Fig. 3.

Corresponding kinetic quantitative data for these reactions of peroxyl radicals with the studied antioxidants are presented below.

The average rates of the methyl linoleate oxidation reaction in the absence and presence of antioxidants at different concentrations are summarised in Table 1, and the oxygen uptake relative rates in the presence of antioxidants compared to the rate of oxygen uptake in the absence of antioxidants are shown in Table 2.

As the folate concentration in the reaction mixture increases, the average rate of the methyl linoleate oxidation reaction decreases (Table 1). According to the data presented in Table 2, there is a weak dependence of the antioxidant activity of folates on an increase in their concentrations. This indicates that FA and its derivatives, DHF, THF and 5-FTHF, exhibit a slightly stronger antioxidant activity at high concentrations.

The rate constants for the reaction of peroxyl radicals with selected folates were determined according to theScheme and the equation [23]. This equation is applicable in experiments in which a clear inhibition period was not detectable. In the case of folates, this equation was performed:

$$\frac{R_0}{R_{AO}} - \frac{R_{AO}}{R_0} = \frac{m_{st} k_{inh} [AO]_0}{\sqrt{2 k_t R_i}}.$$

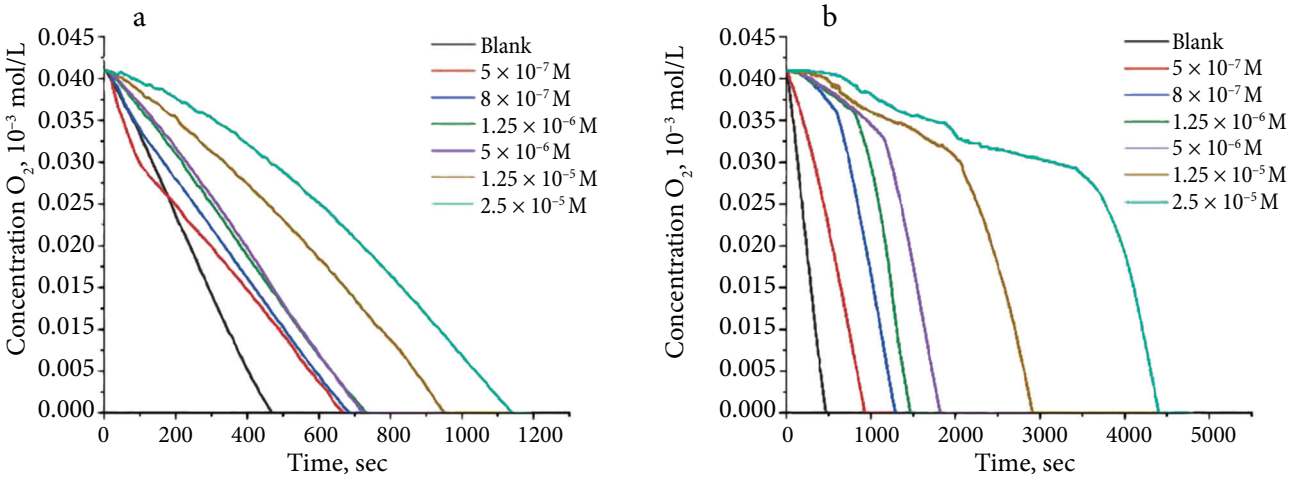


Fig. 2. Kinetic curves of oxygen absorption during the peroxidation of methyl linoleate in the micellar reaction system without (blank) and with the studied antioxidants – FA (a) and trolox (b) at different concentrations: 5×10^{-7} M; 8×10^{-7} M; 1.25×10^{-6} M; 5×10^{-6} M; 1.25×10^{-5} M; 2.5×10^{-5} M. $[AAPH]_0 = 7 \times 10^{-3}$ M; $[ML]_0 = 2.5 \times 10^{-2}$ M; $[Triton\ X\text{-}100]_0 = 5 \times 10^{-2}$ M; $[Phosphate\ buffer]_0 = 5 \times 10^{-2}$ M; $t = 37 \pm 0.1$°C

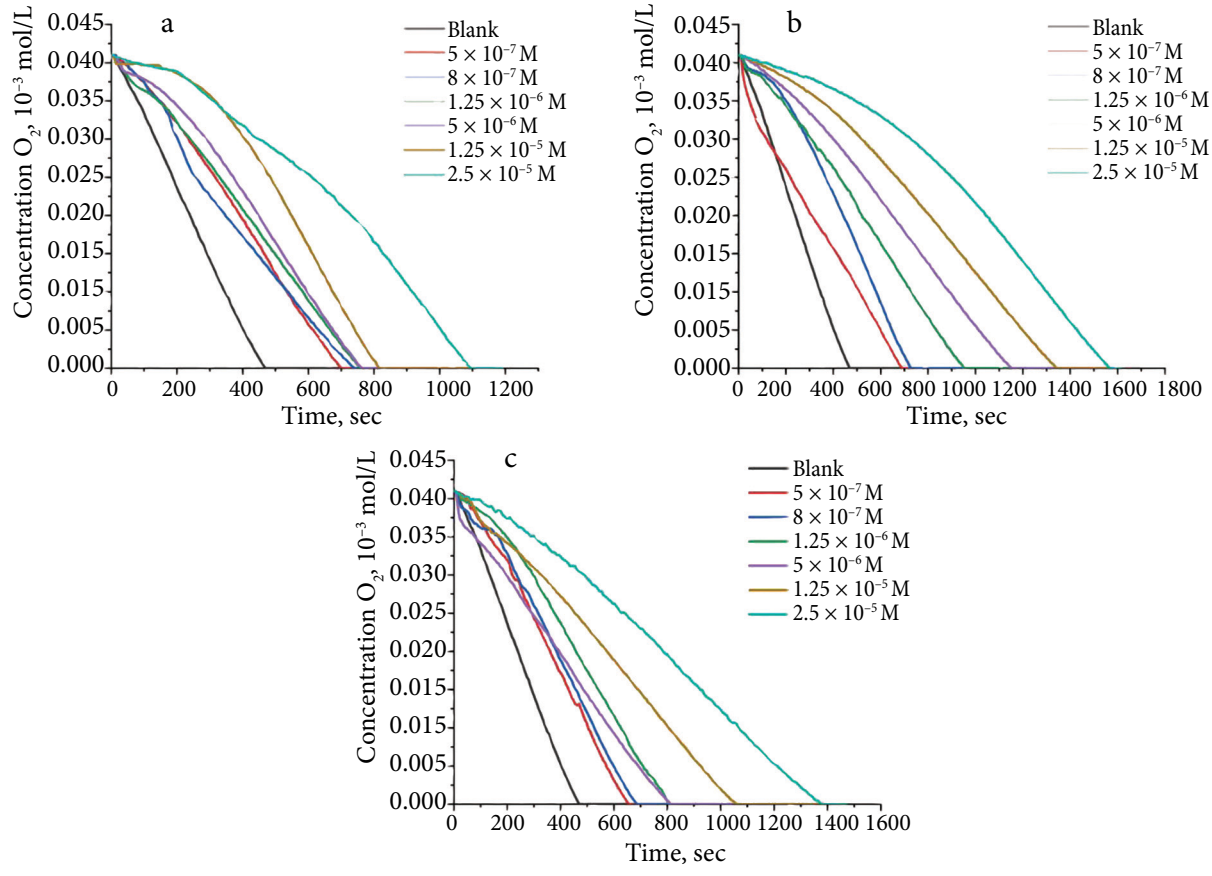


Fig. 3. Kinetic curves of oxygen absorption during the peroxidation of methyl linoleate in the micellar reaction system without (blank) and with the studied antioxidants – DHF (a), THF (b) and 5-FTHF (c) at different concentrations: 5×10^{-7} M; 8×10^{-7} M; 1.25×10^{-6} M; 5×10^{-6} M; 1.25×10^{-5} M; 2.5×10^{-5} M. $[AAPH]_0 = 7 \times 10^{-3}$ M; $[ML]_0 = 2.5 \times 10^{-2}$ M; $[Triton\ X\text{-}100]_0 = 5 \times 10^{-2}$ M; $[Phosphate\ buffer]_0 = 5 \times 10^{-2}$ M; $t = 37 \pm 0.1$°C

Table 1. **The values of the average rates ($\times 10^{-3}$ Msec^{-1}) of the 2.5×10^{-2} M methyl linoleate oxidation reaction in the absence and presence of antioxidants at 37°C**

Antioxidant	Concentration, M					
	5×10^{-7}	8×10^{-7}	1.25×10^{-6}	5×10^{-6}	1.25×10^{-5}	2.5×10^{-5}
Folic acid (FA)	0.061	0.059	0.056	0.051	0.043	0.036
Dihydrofolic acid (DHF)	0.058	0.055	0.048	0.043	0.036	0.028
Tetrahydrofolic acid (THF)	0.059	0.056	0.045	0.041	0.034	0.026
5-Formyl tetrahydrofolic acid (5-FTHF)	0.062	0.059	0.050	0.045	0.038	0.030
Trolox	0.044	0.031	0.022	0.019	0.014	0.009

Note. Reaction average rate in the absence of antioxidants is equal to 0.087×10^{-3} M $\times$ sec^{-1}.

Table 2. **The values of the relative rates of the 2.5×10^{-2} M methyl linoleate oxidation reaction in the absence and in the presence of antioxidants at 37°C**

Antioxidant	Concentration, M					
	5×10^{-7}	8×10^{-7}	1.25×10^{-6}	5×10^{-6}	1.25×10^{-5}	2.5×10^{-5}
Folic acid (FA)	0.701	0.678	0.643	0.586	0.494	0.413
Dihydrofolic acid (DHF)	0.666	0.632	0.551	0.494	0.413	0.321
Tetrahydrofolic acid (THF)	0.678	0.643	0.517	0.471	0.391	0.298
5-Formyl tetrahydrofolic acid (5-FTHF)	0.713	0.678	0.575	0.517	0.437	0.345
Trolox	0.506	0.356	0.253	0.218	0.161	0.104

Here R_0 and R_{AO} are the initial rates of peroxidation without or with an antioxidant, respectively; R_i is the rate of initiation; $2k_t$ is the rate constant for the termination of the oxidative substrate; k_{inh} is the rate constant for the reaction of the peroxyl radical with an antioxidant; m_{st} is a stoichiometric factor for the removal of the peroxyl radical with an antioxidant.

The m_{st} parameter indicates how many peroxidation reactions were kinetically terminated as a result of the reaction between peroxyl radicals and antioxidants. For monophenolic antioxidants (e.g. folates), this stoichiometric coefficient coincides with the antiradical capacity f_R, representing the number of molecules of peroxyl radicals that react with one molecule of the antioxidant under the reaction conditions. In the case of the investigated folates, m_{st} equals 2 for FA, 3 for DHF, 4 for THF and 2.5 for 5-FTHF [24].

The value of the initiation rate (R_i) is proportional to the concentration of the initiator (AAPH) and does not change with the addition of an inhibitor – antioxidant. The kinetics of the chain propagation and termination stages do not depend on the nature of the initiator.

R_0 and R_{AO} are determined from the corresponding kinetic curves of oxygen consumption (Figs. 1 and 2). From the slope of the curve of the blank, the value of R_0 is determined (k_p = 70 M^{-1} s^{-1}). For the determination of R_{AO}, tangents were constructed for the antioxidant curves at the starting points. The value of the slope is equal to R_{AO}. R_i is calculated using the value of k_i (1.07×10^{-6} s^{-1}).

Using experimental values for R_0, R_{AO}, R_i, and [ML], as well as k_t, according to literature data [19], gives the value of k_{inh}. The corresponding values of k_{inh} for the reactions of the peroxyl radicals with the studied antioxidants are presented in Table 3.

The analysis of quantitative kinetic data presented above reveals the sequential changes in the activity of folates. Therefore, we can conclude that the kinetically preferred antiradical/antioxidant activities of the examined folate family can be generally classified in the following order:

FA < 5-FTHF < THF ≅ DHF.

This sequence of changes in the antiradical/antioxidant reactivity of folates is generally consistent with the experimental results presented in Ref. [24]. Reduced forms of folic acid – DHF and THF – have been found to have a significantly greater activity compared to FA and 5-FTHF in Oxygen Radical Absorbance Capacity (ORAC) and DPPH assays.

CONCLUSIONS

The antiradical/antioxidant properties of folates – FA and its conformational structural forms (DHF, THF and 5-FTHF) – were determined in the chain oxidation reaction of methyl linoleate in micellar aqueous solutions. Based on the experimental data obtained, it was shown that the studied folates exhibit antioxidant properties and act as 'scavengers' of free radicals in the peroxidation reaction of methyl linoleate. They can serve as antioxidant regulators of lipid peroxidation in cell membranes, functioning through the mechanism of kinetic chain termination at the cell membrane – water interface.

According to the quantitative kinetic parameters, the increasing order of the antiradical/antioxidant reactivities of folates has been revealed.

Table 3. **The values of k_{inh} ($\times 10^4$ $M^{-1}sec^{-1}$) obtained for folates for the thermally initiated peroxidation of 2.5×10^{-2} M methyl linoleate with 7×10^{-3} M AAPH ($R_i = 7.49 \times 10^{-9}$ $Msec^{-1}$) at 37°C**

Antioxidant	Concentration, M					
	5×10^{-7}	8×10^{-7}	1.25×10^{-6}	5×10^{-6}	1.25×10^{-5}	2.5×10^{-5}
Folic acid (FA)	11.64	8.31	7.47	2.17	1.31	0.99
Dihydrofolic acid (DHF)	21.71	14.29	13.33	4.62	2.98	2.15
Tetrahydrofolic acid (THF)	30.38	21.94	21.00	7.61	4.52	3.19
5-Formyl tetrahydrofolic acid (5-FTHF)	16.28	11.15	10.55	3.22	1.77	1.34

$R_0 = 1.94 \times 10^{-7}$ $Msec^{-1}$; $k_t = 3 \times 10^3$ $M^{-1}sec^{-1}$.

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FOLATŲ POVEIKIS METILO LINOLEATO OKSIDACIJOS REAKCIJAI MICELINIUOSE TIRPALUOSE