ANTIOXIDANT CO-ACTIONS OF ASCORBIC AND DIHYDROXYFUMARIC ACIDS INVESTIGATED BY EPR SPECTROSCOPY

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Abstract. The intricate dynamics of antioxidant interactions holds promise for innovating formulations to reduce patient antioxidants doses and prolong efficacy, these aspects being also important for other industrial applications, such as food preservation. In this context, the study presents data on the antioxidant interaction between ascorbic (AA) and dihydroxyfumaric acids (DHF) determined *via* DPPH· method, by applying EPR spectroscopy. Two calculations methods used demonstrated strong and moderate synergistic effects, with antioxidant interaction parameter (AI) of 1.24 and 0.9, respectively. The type of antioxidant interaction is dependent on the concentration ratio of the ascorbic and dihydroxyfumaric acids, thus, at the mM DHF/mM AA ratios of 1.4 and 1.7 the highest synergistic effects with AI of 1.24 have been noticed, but at the mM DHF/mM AA ratio of 1 – an antagonistic effect with AI of 0.93 was registered.

Introduction

The interaction between antioxidants in terms of antioxidant activity can be synergistic, additive or antagonistic [1], the synergistic interactions being of most interest for science and industries due to the advantages that they can offer: increased efficacy, reduced amount of antioxidants needed, replacement of synthetic antioxidants [1]. According to the reported data, there are several mechanisms of mutual antioxidant interaction that can generate synergistic, additive or antagonistic effects: (1) the regeneration processes, (2) formation of antioxidants` intermolecular complexes, dimers or adducts, and (3) complementary effects that presume the effect of the solvent, pH, concentration, solubility *etc.* [2,3].

The antioxidant interaction between ascorbic acid and other antioxidant compounds has been recently reported in the literature [2,4]. Strong synergistic effects have been found for different concentrations of ascorbic acid (AA) and *trans*-aconitic acid assessed by DPPH· assay [5]. In combination with polyphenols, ascorbate has flavonoid-protective and flavonoid enhancing antioxidant activities [6]. AA regenerates quercetin and catechin from their oxidised forms, *o*-quinones [6]. By employing the Co(II)-EDTA

luminol chemiluminescence method, Choueiri, L. *et al.* found that the mixture of quercetin and AA has the highest antioxidant activity at the ratio 2:1 [7]. The concentration of AA is equally important for the type of antioxidant interaction parameter (AI). Different catechin – AA ratios are attributed to the formation of two distinct oligomeric structures, and, consequently, to different antioxidant outcomes [8]. At lower AA concentrations, the catechin oligomerization and formation of procyanidin structures is noticed, which determine the enhancement of the antioxidant behaviour of the mixture [8]. In the solutions of AA and *O*-glucosylated flavonoids, rutin or naringin, synergistic effects are observed, unlike the case of AA and non-*O*-glucosylated flavonoids [9]. Lo Scalzo, R. successfully applied the EPR spectroscopy to investigate the AI of AA, chlorogenic acid and cysteine in presence of glucose or citric acid, by using superoxide anion, hydroxyl radical and peroxyl radical [10]. Mainly additive effects have been found for these combinations [10].

While AA is one of the most studied natural antioxidant, dihydroxyfumaric acids (DHF) has gained high scientific interest in recent years due to its role in the "glyoxylate scenario" of primordial

metabolism [11,12]. Also, DHF is a constituent of the cycle of dicarbonic acids – the Baroud' cycle of tartaric acid and its intermediate products' transformation to oxalic acid [13]. Thus, both compounds are present in various natural sources and could easily interact [13,14].

In our recent studies, the improvement of the total antioxidant activity when mixing AA and DHF has been reported [15]. By employing the Stopped-Flow technique, the antioxidant activity of single compounds and their mixtures have been determined *via* DPPH• assay in 98% ethanol and wine simulated matrix [15]. Data obtained after 2 seconds of single antioxidant and free radical interaction demonstrated that in wine simulated matrix the DHF's observed rate constants are 10 times higher than in ethanol, and 2 times higher in case of AA [15]. By combining AA and DHF, the results revealed a decrease of the observed rate constants [15], however, because within 2 seconds the reaction did not reach the steady state, further investigations are needed to formulate a proper conclusion regarding the type of antioxidant interaction between the two antioxidants.

The importance of this investigation relies on the application potential of synergistic effect of the antioxidants in science and industries, as long as the multicomponent systems similar to those found in foods have the capacity to act through multiple mechanisms of action and to inhibit oxidation at many different stages [16]. Therefore, the aim of this work was to investigate the type of AI between AA and DHF with the DPPH• assay, by applying the EPR spectroscopy.

Experimental
Materials

The following reagents and solvents were used in the research: *L*-ascorbic acid (AA), >99%, dihydroxyfumaric acid (DHF), 98%, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) were purchased from Sigma-Aldrich, Germany; the solvent – ethanol (EtOH), 96%, was purchased from Mic-Tan, Republic of Moldova. Reagents and solvents were used without further purification.

Methods

The antioxidant activity was determined using the DPPH• test [17]. Stock solutions of each antioxidant (130 μM) were daily prepared in 98% EtOH. The concentration of the DPPH• was checked before every series of experiments to correspond to 0.05 mg/mL initial concentration. Each solution was sonicated for 5 minutes for a complete dissolution of antioxidants or free radical. The DHF/AA millimolar concentration ratios needed to perform the reactions were as follows: 0.5, 0.6, 0.7, 1, 1.4, 1.7 and 2. The reaction mixtures consisted of equal parts (0.5 mL) of the antioxidants (single compound or mixture of antioxidants) and DPPH•. The reaction time was of 30 min in the dark, to reach the steady stare redox reaction. Each sample was prepared in triplicate.

Equations and formulas

The half-maximal efficient concentration (EC_{50}) parameter is defined as the concentration of antioxidant required to annihilate 50% of the free radicals, and is expressed as mole of antioxidant per mole of DPPH• (mole AOX/mole DPPH•). To determine EC_{50}, first, the percentage of the remaining DPPH• (% rem. DPPH•) at the steady state was calculated according to the Eq.(1); afterwards, the results obtained for each sample were plotted against the mole AOX/mole DPPH• ratio.

$$\% \text{ rem. DPPH}^{\bullet} = \left(\frac{A_{sample}}{A_{control}}\right) \times 100 \quad (1)$$

where, A_{sample} is the absorbance of the sample at the steady state;
$A_{control}$ is the absorbance of the sample at the time zero.

To find the type of AI, the calculation method described previously [5,18] was applied. First, the inhibition percentage (%I) was calculated by using the Eq.(2), this parameter being further used for establishing the AI.

$$\% \text{I} = 1 - \left(\frac{A_{sample}}{A_{control}}\right) \times 100 \quad (2)$$

The AI parameter of a mixture was calculated from the ratio of the experimental value of the inhibition percentage of the mixture (%$I_{mixture}$) and the theoretical value (%$I_{theoretical}$), Eq.(3).

$$\text{AI} = \left(\frac{\% I_{mixture}}{\% I_{theoretical}}\right) \quad (3)$$

where,

$$\% \text{I}_{theoretical} = \% I_{AA} + \% I_{DHF} - \frac{\% I_{AA} \times \% I_{DHF}}{100} \quad (4)$$

where, $\% I_{AA}$ is the inhibition percentage of AA, tested alone in reaction with DPPH•, Eq.(4);
$\% I_{DHF}$ is the inhibition percentage of DHF, tested alone in reaction with DPPH•, Eq.(4).

Therefore, a synergistic effect is found when the AI> 1; if AI= 1, then the interaction is additive; and a AI< 1 reveals an antagonistic effect [5,18].

Another method to calculate the type of AI is by determining the fractional inhibitory concentration (FIC) [19,20]. The FIC index is calculated by summing the characteristic FIC values of each tested compound. For this it is necessary to know the EC_{50} value for each compound tested individually and in combination.

The FIC calculation for the AA and DHF antioxidant interaction was performed according to Eqs.(5-7), the FIC index for each compound being determined by dividing the EC_{50} value of the combination of antioxidants to the EC_{50} value for the individual compound.

$$FIC_{INDICE}= FIC_{AA} + FIC_{DHF} \qquad (5)$$

$$FIC_{AA}= \frac{EC_{50}\ (of\ AA\ in\ presence\ of\ DHF)}{EC_{50}\ (of\ AA\ alone)} \qquad (6)$$

$$FIC_{DHF}= \frac{EC_{50}\ (of\ DHF\ in\ presence\ of\ AA)}{EC_{50}\ (of\ DHF\ alone)} \qquad (7)$$

where, FIC_{AA} is the fractional inhibitory concentration for AA;

FIC_{DHF} is the fractional inhibitory concentration DHF.

In the case of the FIC index, a synergistic effect is found when FIC< 1; if FIC= 1, then the interaction is additive; and a FIC> 1 demonstrates the presence of an antagonistic effect.

Instruments

The samples were inserted into a standard rectangular cavity of an EMX X-band EPR spectrometer (Bruker, Germany) operating at 9.8 GHz with a modulation frequency of 100 kHz. Spectra were recorded at r.t. (25°C) using the following parameters: center field 3490 G, sweep width 100 G, receiver gain 40 dB, modulation amplitude 5 G, attenuation 10 dB (20000 mW), time constant 40.96 ms. The EPR spectrum was registered right after 30 minutes of reaction.

The data obtained were analysed with ANOVA and Student's *t* tests to evaluate the statistical significance of the difference between the means using the Microsoft Excel programme. A *p* value of 0.05 was considered significant.

Results and discussion

EPR spectroscopy is a technique used to investigate the radical species present or formed in the chemical reactions, therefore the use of the EPR spectroscopy is appropriate for investigating antioxidant activity and interactions between AA, DHF and DPPH$^•$. The Figure 1 illustrates the spectra for 127 μM DPPH$^•$, and 65 μM of AA or DHF. Of the three compounds, DPPH$^•$ is the only one that offers detectable by the EPR spectroscopy signals; its spectrum is similar to the previous ones reported in the literature [21]. From the same figure, one can notice that the antioxidants AA and DHF do not generate any signals, meaning that these compounds are not in a free radical form.
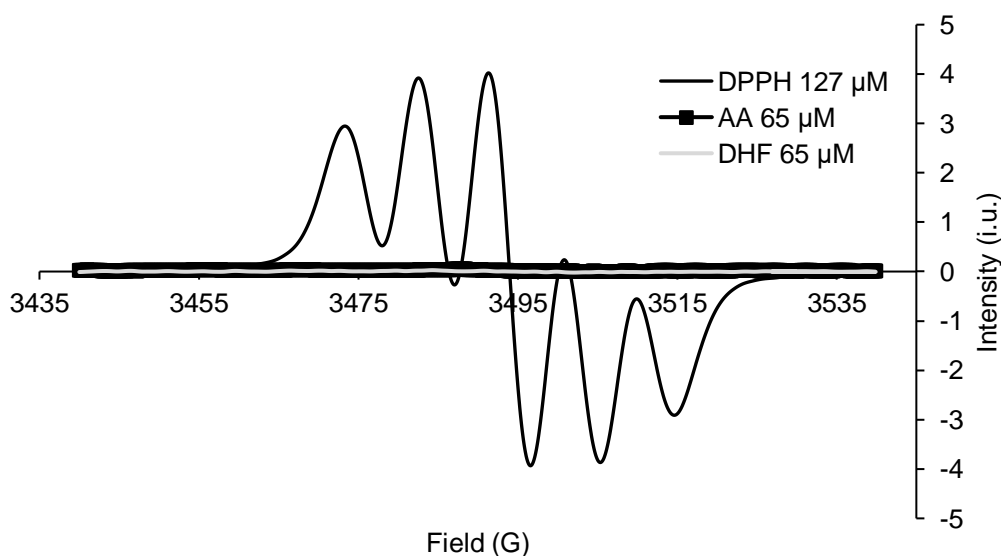

Figure 1. EPR spectra for 127 μM DPPH$^•$, 65 μM AA and 65 μM DHF.

After the interaction with different concentrations of AA or DHF, the intensity of the DPPH$^\bullet$ signal decreases as a consequence of the antioxidant activity of the acids (Figure 2). It was established that in these experimental conditions, 50% of radical species were annihilated by 0.24±0.00 moles of AA and 0.18±0.00 moles of DHF (Figure 2). The EC$_{50}$ values determined correspond to the previously reported results [22,23] and highlight the ability to annihilate free radicals more accentuated with DHF than AA, this property being similar to the antioxidant capacity. The EPR data reflect a direct relationship between the signal intensity and the concentration of the free radical in the system, thus, the data in Figure 2 demonstrate the total annihilation of DPPH$^\bullet$, unlike the UV-Vis method which is influenced by the yellowish colour of the solution after the scavenging the DPPH$^\bullet$.

To determine the type of antioxidant interaction between AA and DHF, several concentration ratios of the antioxidants in the reaction with DPPH$^\bullet$ were analysed, as illustrated in Figure 3.

Among the seven investigated cases, the mM DHF/mM AA ratios of 1.4 and 1.7 demonstrated the strongest synergistic effect with AI of 1.24, and high antioxidant activity (Figure 3).
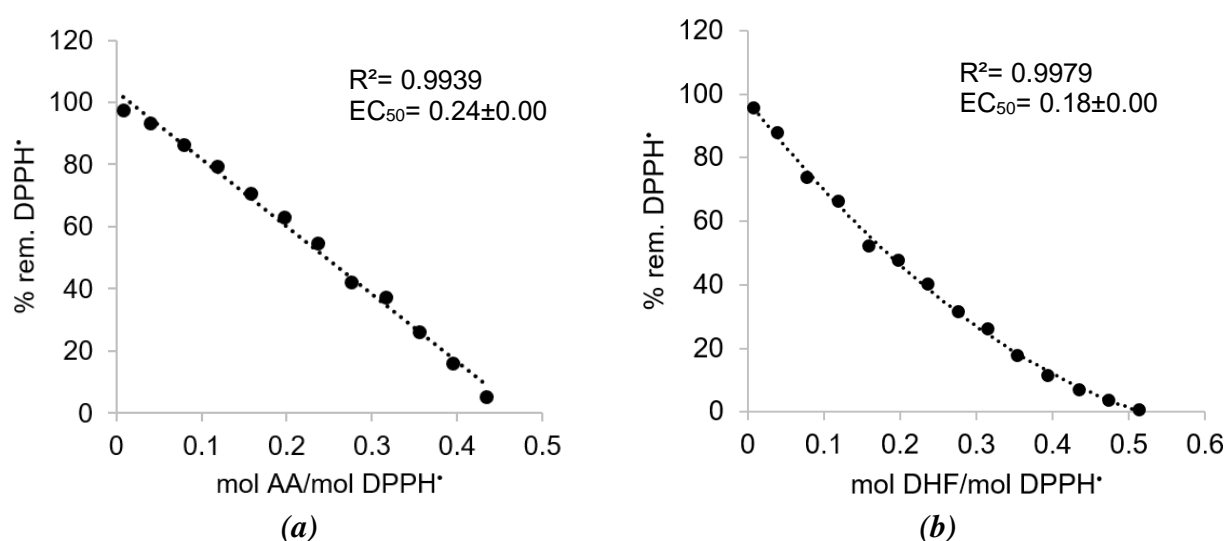

Figure 2. Graphical representation of the dependence of the % remaining DPPH$^\bullet$ on the antioxidant/DPPH$^\bullet$ molar ratio for reactions: AA - DPPH$^\bullet$ (linear fitting) *(a)* and DHF - DPPH$^\bullet$ (exponential fitting) *(b)*.
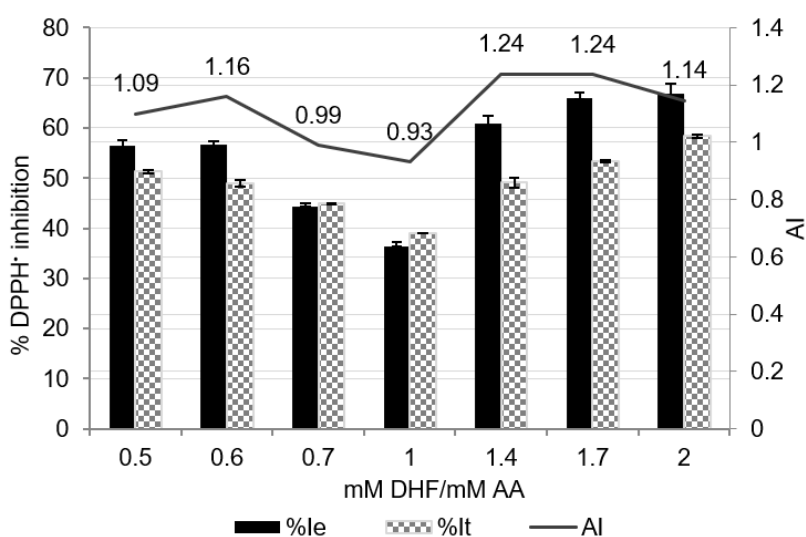

Figure 3. Representation of the %DPPH$^\bullet$ inhibition determined experimentally (black) and theoretically calculated (plaid pattern) (left axis). Representation of the type of antioxidant interaction for each mM DHF/mM AA ratio (right axis). Data are presented as mean values (n≥ 3). Significant difference ($p < 0.05$) to 1.24 are calculated using one-sample Student's *t* test.

The ratios mM DHF/mM AA= 1 gave the weakest result of 0.93, being characteristic of an antagonistic interaction. This fact is also observed by analysing the experimental and theoretical DPPH$^\bullet$ inhibition percentages (Figure 3). Thus, for DHF/AA concentration ratios of 0.7 and 1, the theoretically calculated radical inhibition is higher than the one determined experimentally (Figure 3). At the same time, in the samples with the highest synergism, the %DPPH$^\bullet$ inhibition established in the mixture is higher than the theoretical one (Figure 3). Additionally, a synergistic effect can be observed at the 0.5 and 0.6 mM DHF/mM AA ratios, although this effect is of a lower magnitude compared to the synergistic interaction between AA and DHF at the 1.7 ratio.

Using the same calculation method, Piang-Siong *et al.* reported similar synergistic effects between natural antioxidants (AA, caffeic acid, gallic acid) and *trans*-aconitic acid [5]. The highest synergistic effect of 1.24 was found for the combination of gallic and *trans*-aconitic acids; in the samples with AA and *trans*-aconitic acid the largest synergistic effect recorded was of 1.15 [5]. The data reported demonstrate the importance of the compounds' concentration on the amplitude of the synergistic interaction [5].

The concentration of the tested compounds is equally important for the total antioxidant activity of the mixture. At the mM DHF/mM AA ratio of 2, where the concentration of DHF is twice larger than that of AA, the %I$_e$ is the highest – 66.8%; on the contrary, at the mM DHF/mM AA ratio of 0.5, the inhibition of the DPPH$^\bullet$ is diminished – 56.4%, and the lowest %I$_e$ of 36.4% is found for the ratio 1.

Finding the ratio mM DHF/mM AA= 1.7 to be the one that possesses the strongest synergistic effect and higher antioxidant activity, the dependence of the synergistic effect on the total concentration of antioxidants was further investigated, respecting in all samples the ratio mM DHF/mM AA= 1.7 (Figure 4).

This fact allowed the determination of the FIC index, and to confirm the presence of the synergistic AI between DHF and AA. The given method is often used in biochemistry and pharmacology to determine synergistic/antagonistic interactions between medicinal preparations [24], it is also used in microbiology to evaluate synergistic effects between antimicrobial agents [19].

Figure 4 demonstrates that by increasing the antioxidants' concentration (but maintaining the mM DHF/mM AA ratio 1.7), the total antioxidant activity of the mixture increases exponentially. Using Eqs.(5-7), the FIC index for the ratio mM DHF/mM AA= 1.7 was determined to be 0.9, a value that describes a moderate synergistic interaction between the two antioxidants.

Data obtained by the EPR method are consistent with our previous UV-Vis results, which demonstrate the synergistic effect between AA and DHF [25], showing that once the concentration of both compounds increases, the total antioxidant activity improves and the synergistic effect increases.
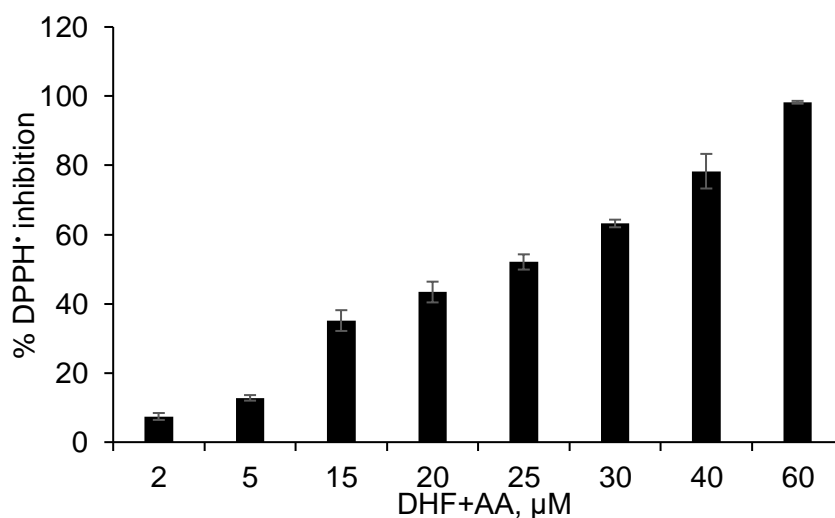
Figure 4. Dependence of the DPPH$^\bullet$ inhibition percentage on the total concentration of antioxidants (DHF+AA, µM) combined in the ratio mM DHF/mM AA= 1.7; Inset: fitting for the exponential model. Data are presented as mean values (n≥ 3).

The synergistic effect is frequently reported to occur when the more efficient antioxidant regenerates the less efficient one [9], taking into account the oxidation potentials [16], which is in agreement with our results. Although both AA and DHF are effective antioxidants, the EC_{50} values indicated that DHF has a slightly higher antioxidant capacity than AA. The study findings revealed that when antioxidants are combined in equimolar proportions, an antagonistic interaction occurs, indicating that such mixtures are not advisable for practical applications. On the other hand, when the molar ratio between DHF and AA exceeds unity, a synergistic antioxidant interaction emerges, which suggests that this interaction is directly supported by the presence of high amounts of DHF, highlighting its pivotal role in this process. The manifestation of synergism when the component with higher antioxidant capacity is predominant suggests alignment with the previously proposed concept, wherein the most effective antioxidant (DHF) regenerates the least effective one (AA). The highest synergistic effect is observed at a larger concentration of DHF compared to AA due to its involvement in competitive reactions, wherein it reduces free radicals and facilitates the regeneration of AA. On the other hand, at the 0.5–0.7 mM DHF/mM AA ratios, where the concentration of AA is the largest, the synergistic effects are lower (1.09 and 1.16). It can be hypothesised that AA also regenerates DHF, but with a lower efficiency, which is in agreement with the data on its antioxidant activity and EC_{50} values (Figure 2). Therefore, analysing Figure 3, it can be seen that the interaction of DHF with AA does not lead to an unequivocal result. Instead, the outcome depends on the deviation from the equimolar ratio of the two antioxidants, with the synergistic effect being linked to the excess of one over the other. Specifically, a synergistic interaction is observed when the DHF/AA ratio deviates from 1:1 by approximately ±0.4 mM/mM and this interaction is more pronounced when DHF is in excess. It appears that DHF has a greater ability to regenerate AA than vice versa, which is consistent with the superior antioxidant activity of DHF compared to AA.

At this stage, it is a challenge to trace a reaction path that describes their antioxidant interaction mechanism at the molecular level. On the other hand, recent findings show that the solutions consisting of AA and DHF in EtOH possess relatively high acidity [25], characterized by the predominance of the *keto-* form of DHF [26], which can cause the partial decarboxylation of DHF. Therefore, further investigations using structural characterization methods (NMR, FTIR, MS *etc.*) are needed to elucidate the mechanism of synergistic interactions of AA and DHF at the molecular level.

Conclusions

By using EPR spectroscopy and two calculation methods, pronounced and moderate synergistic effects between AA and DHF have been demonstrated, as well as antagonistic and additive interactions. The type of antioxidant interaction depends on the concentration ratio of the antioxidants, the highest synergistic effect (1.24) being noticed for the mM DHF/mM AA ratios of 1.4 and 1.7, but an antagonistic effect (0.93) at the ratio 1. The FIC method confirmed the presence of synergistic effects (0.9) of AA and DHF. Studies have indicated that the DHF/AA ratio plays a pivotal role in the manifestation of synergism, occurring in the excess of one antioxidant over the other. Therefore, the alignment between the slightly superior antioxidant activity of DHF over AA and the superunitary molecular ratio (1.7), at which the highest synergistic antioxidant interaction was established, implies that this interaction occurs *via* the regeneration of AA by DHF. The synergistic interaction between DHF and AA is also observed when AA is in excess compared to DHF, though to a lesser extent.

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