Synthetic organic food colouring agents and their degraded products: effects on human and rat cholinesterases

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ABSTRACT

Most synthetic coloured additives are carcinogenic, teratogenic and cause allergic reactions. In this study, the effects of synthetic azo dyes (sunset yellow FCF and carmoisine), as well as their degraded products (sulphanilic acid and naphthionic acid), on both true and pseudo-cholinesterases (ChEs) are studied. The results indicate that the synthetic azo dyes and their degraded products inhibit both human true and pseudo-ChE activities *in vitro*. The concentration of coloured additive that cause 50% inhibition (IC_{50}) and enzyme inhibitor dissociation constant (K_i) show that sunset yellow FCF produces greater inhibition of both true and pseudo-ChEs than does carmoisine and sulphanilic acid, while naphthionic acid produces greater inhibition of pseudo-ChE only. K_i indicates that the affinity of sulphanilic acid for both true and pseudo-ChEs is higher than the other three inhibitors. Inhibition of both true and pseudo-ChEs by sunset yellow FCF is of mixed (competitive and non-competitive) type, but carmoisine and sulphanilic acid are non-competitive. Naphthionic acid produces a competitive inhibition kinetic with plasma ChE only. This inhibition is abolished by dialysis, indicating that their effects are reversible. The effects of sunset yellow FCF, carmoisine, sulphanilic acid and naphthionic acid on rat true and pseudo-ChEs are investigated. The data clearly show that there is a significant decrease in enzyme activity. Sulphanilic acid and sunset yellow FCF are the most potent *in vivo* inhibitors of true ChE and pseudo-ChE, respectively.

KEY WORDS: Carmoisine. Cholinesterases. Naphthionic acid. Sulphanilic acid. Sunset yellow.

Introduction

Food additives are classified according to their function and usage and may be preservatives, antioxidants, colours, flavours, sweeteners, emulsifiers, acidulates, vitamins or minerals.[1] According to US Food and Drug Administration (FDA), there are two classes of colour additives: natural colours and artificial colours.[2] During production, nearly all artificial colours are treated with sulphuric acid or nitric acid, both of which are often contaminated with arsenic.[3]

Most synthetic colour additives are carcinogenic, teratogenic and can cause allergic reactions.[4] Water soluble dyes are biotransformed by microorganisms in the intestinal tract and by azo reductases in the liver, and the toxicity, mutagenicity and carcinogenicity of these dyes in the intestine or liver may be attributed to their metabolites.[5]

The synthetic dyes sunset yellow FCF and carmoisine are classified as mono azo dyes, while their degraded products sulphanilic acid and naphthionic acid are classified as sulphonated aromatic amines. The maximum acceptable daily intake (ADI) of sunset yellow FCF is up to 2.5 mg/kg of bodyweight;[6] however, it can cause reproductive problems and have neurobehavioural effects,[7] and has been shown to inhibit liver mitochondrial respiration by 28%.[8] Significant increases in serum total lipids, cholesterol, triglycerides, total proteins, globulin and serum transaminases have been observed in rats whose diet was supplemented with various concentrations of chocolate colours, sunset yellow FCF, tartarazine, carmoisine and brilliant blue.[9] The maximum ADI for carmoisine is up to 4 mg/kg of bodyweight.[10]

Solutions of these coloured dyes are not stable in sunlight or in the dark, particularly when common food acids (e.g., citric acid and ascorbic acid) are present,[11] which significantly enhance the degradation of both sunset yellow FCF and carmoisine. The degraded products from sunset yellow FCF are sulphanilic acid and the sodium salt of 2-naphthalene-sulphonic acid-5-amino-6-hydroxyl. The degraded products from carmoisine are 1-naphthelene-sulphonic acid-4-amino-sodium salt (naphthionic acid and the sodium salt of 1-naphthalene-sulphonic acid-3-amino-4-hydroxyl. These aromatic amines are not licensed as food additives and some are reported to possess health hazards.[11]

Sulphanilic acid and its N-acetylated derivatives interact with rat liver glutathione S-transferase (GST) isoenzymes (AA, A, B, C, E, and M) by direct binding to protein, and this leads to inhibition.[12] Cholinesterases are a group of enzymes that degrade the esters of choline and play a role in neurotransmission in the autonomic and somatic motor nervous systems, and their inhibition results in accumulation of acetylcholine.[13] Recent work[14] indicates that sunset yellow FCF, quinoline yellow and erythrosine inhibit

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Table 1. Kinetic constants characterising inhibition of plasma and erythrocyte (RBC) cholinesterases by sunset yellow FCF, carmoisine, sulphanilic acid and naphthionic acid.

Inhibitor	K_i x 10^{-4} mol/L		K_v/K_m	
	Plasma ChE	RBC AChE	Plasma ChE	RBC AChE
Sunset yellow FCF	2.8 ± 0.16 $^{\$\dagger@*}$	0.75 ± 0.05$^{\$\ddagger}$	2.11 ± 0.08$^{\$\dagger@}$	0.45 ± 0.02$^{\$\ddagger}$
Carmoisine	3.25 ± 0.16$^{\$@\dagger *}$	1.90 ± 0.17$^{\$\dagger}$	2.44 ± 0.09$^{\$@\dagger *}$	1.15 ± 0.03$^{\$\dagger}$
Naphthionic acid	56 ± 0.2$^{\$\ddagger\dagger}$	—	42 ± 2.05$^{\$\ddagger\dagger}$	—
Sulphanilic acid	80 ± 1.5$^{\dagger@\dagger *}$	140 ± 3.2‡†	60 ± 1.90$^{\dagger@\dagger *}$	84.3 ± 1.44‡†

Results expressed as mean ± SEM.

* Significant difference to corresponding value of erythrocyte cholinesterase.

† Significant difference to corresponding value using sunset yellow FCF.

‡ Significant difference to corresponding value using carmoisine.

§ Significant difference corresponding value using sulphanilic acid.

@ Significant difference to corresponding value using naphthionic acid

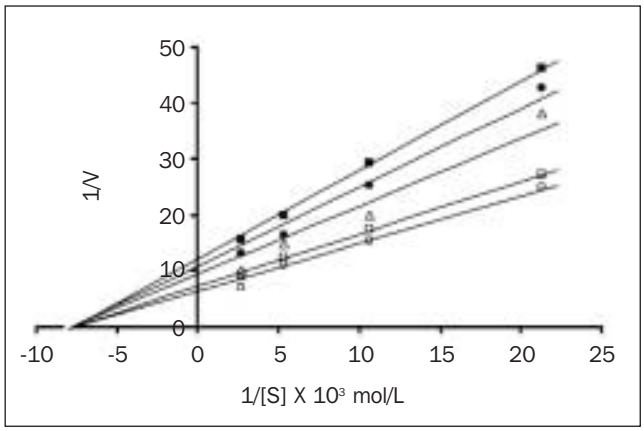

Fig. 1. Lineweaver-Burk plot of human plasma ChE inhibited by sulphanilic acid. (○) Control, (□) 17.70, (△) 35.40, (●) 53.10 and (■) 79.60 x 10^{-4} mol/L. Each point represents the average value of triplicate determinations from a typical experiment.

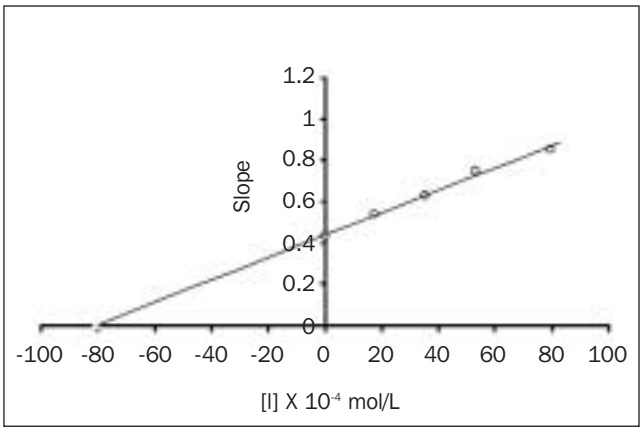

Fig. 2. Cleland replot of the slopes obtained from Figure 1 against the inhibitor concentration [I].

human erythrocyte and plasma ChE *in vitro*.

The aim of this study is to evaluate the possible biochemical effects of sunset yellow FCF and carmoisine, and their by-products (the sulphonated aromatic amines naphthionic acid and sulphanilic acid), on human ChE and rat (*in vivo*) true and pseudo-ChE through the determination of kinetic parameters that reflect the interaction between colouring agent and enzyme.

Materials and methods

Reagents

Sunset yellow FCF (E110, colour index [CI] 15985), the disodium salt of 1-(4 sulphophenylazo)-2-naphthol-6-sulphonic acid; carmoisine (E122, CI 1470) the disodium salt of 2-(4-sulpho-1-naphthylazo)-1-naphthole-4-sulphonic acid; sulphanilic acid, *P*-amino benzen sulphonic acid; and naphthionic acid, 1-naphthalene sulphonic acid-4-amino-sodium salt (all obtained from Ellis and Everard, UK). Acetylthiocholine iodide (AthChI) and 5,5′ dithiobisnitrobenzoic acid (DTNB) were obtained from the Sigma Chemical Company. All other chemicals used were reagent grade.

Subjects

Local ethics committee approval was obtained. The study comprised 10 healthy male subjects (for *in vitro* study) (age range: 25–35 years old; red cell count 4.2–5.8 x 10^{12} cell/L). The study was explained to all participating subjects and written, informed consent was obtained in each case. Laboratory investigations including complete blood picture, liver and kidney profiles, and complete urine analysis.

In vitro studies

Fresh heparinised blood was taken from each subject. Plasma (pseudo) ChE was obtained by centrifugation of 1 mL heparinised blood at 500 x*g* for 10 min, and then diluted (1 in 200) with 0.1 mol/L phosphate buffer (pH 8.0). Erythrocyte (true) ChE was obtained by adding 10 µL red cells to 5 mL saline and then centrifuging the mixture 500 x*g* for 10 min. The upper layer was discarded and the remaining erythrocytes washed (x2) with 5 mL saline. The erythrocytes were resuspended in 0.1 mol/L phosphate buffer (pH 8.0) at a dilution of 1 in 500 (7.8 x 10^6 – 9.5 x 10^6 cell/mL).[15]

Table 2. Effect of dialysis on human plasma cholinesterase (ChE) activity before and after the addition of sunset yellow FCF, carmoisine, suphanilic acid and naphthionic acid, using the IC_{50} of each inhibitor.

Inhibitor	[I] x 10^{-4} mol/L	% ChE activity	
		Before dialysis	After dialysis
Sunset yellow FCF	3.30	52.0 ± 2.2	88.00 ± 2.8*
Carmoisine	3.38	51.5 ± 1.9	93.39 ± 1.3*
Sulphanilic acid	129	50.2 ± 1.7	75.50 ± 1.1*
Naphthionic acid	25	49.8 ± 1.8	90.40 ± 2.7*

Results expressed as mean ± SEM (n = 5).

* Significant difference to corresponding values before dialysis (P<0.001).

Table 3. Plasma and erythrocyte (RBC) cholinesterase activities of rats fed a diet containing sunset yellow FCF, carmoisine, sulphanilic acid or naphthionic acid.

Group	Plasma ChE activity U/L	RBC AChE activity μmol/min per 10^6 RBCs
Control	4494 ± 108	0.173 ± 0.021
Sunset Yellow FCF	3463 ± 258*	0.148 ± 0.009*
Carmoisine	3805 ± 115*	0.135± 0.014*
Sulphanilic acid	3915 ± 169*	0.120± 0.006*
Naphthionic acid	3664 ± 101*	0.173± 0.014

Results expressed as mean ± SEM (n=5).

* Significant difference to corresponding control value (P<0.05).

The effects of sunset yellow FCF, carmoisine, sulphanilic acid and naphthionic acid on the activities of both plasma and erythrocyte ChE were measured in triplicate at 37°C by the colorimetric method of Ellman *et al*.[16] at 412 nm.

Separate assay mixtures were prepared of 3 mL plasma suspension (diluted 1 in 200) or 3 mL erythrocyte suspension (diluted 1 in 500) in 0.1 mol/L phosphate buffer (pH 8.0), containing an amount of enzyme equivalent to 0.08 U/mL, 100 μL 0.1 mol/L DTNB and 20 μL 0.5 mmol/L AThChI. Control ChE activity was regarded as 100 % (in absence of inhibitor) and enzyme activity after the addition of each inhibitor was expressed as a percentage of the control activity.

To determine inhibition type, the enzyme-inhibitor dissociation constant (K_i) of each colouring agent (or their degraded products) and the Michaelis constant (K_m), the assay was carried out with inhibitor present at a constant concentration and substrate (AthChI) concentrations of 0.47, 0.94, 1.875, 2.25, 3.00 or 3.75 x 10^{-4} mol/L.

Each colouring agent (or its degraded product) was added to the above mixture in the following concentrations: sunset yellow FCF at 8.80, 12.71, 16.26, 19.53 or 22.56 x 10^{-5} mol/L; carmoisine at 8.80, 12.71, 16.26 or 19.53 x 10^{-5} mol/L; sulphanilic acid at 8.8, 35.4, 53.1 or 79.6 x 10^{-4} mol/L and naphthionic acid at 17.7, 35.4, 53.1 or 79.6 x 10^{-4} mol/L. The enzyme-inhibitor mixture and enzyme alone (controls) were also assayed.[16]

The K_i/K_m (affinity) values for true and pseudo-ChE inhibition were calculated. Measurement of the concentration required to inhibit 50% of enzyme activity (IC_{50}) may be considered to be a parameter that reflects the differences in inhibitory power of these dyes and their degraded products.

Dialysis

Five cellophane tubes each containing 3 mL plasma suspension and an amount of enzymes equivalent to 0.08 U/mL were used. A different inhibitor was added to each tube at the IC_{50} concentration: sunset yellow at 3.3 x 10^{-4} mol/L, carmoisine at 3.38 x 10^{-4} mol/L, sulphanilic acid at 129 x 10^{-4} mol/L and naphthionic acid at 25 x 10^{-4} mol/L. Control tubes were prepared without inhibitor. The tubes were left to dialyses against 0.1 mol/L phosphate buffer (pH 8.0) at 4°C overnight, and were then assayed for ChE activity as described previously.[14]

In vivo studies

In vivo study involved the use of 25 male albino rats (125–150 g), supplied by Medical Research Institute animal house, Alexandria University. They were housed in groups (five per cage) and given free access to food and tap water. The animals were placed in metabolic cages two weeks prior to the beginning of the study and were fed a standard diet[17] until their weight reached 200 g (red cell count 4.9–6.2 x 10^{12} cell/L). Control animals continued on the standard diet and the experimental groups were fed the same diet supplemented with 0.4 g of each colouring agent (or degraded product) per kg of feed.[17,18]

The food intake of each rat was calculated as the difference between the amount of food given and the amount left behind each day during the entire period of study. Each rat consumed 2 g/day (containing 4 mg/kg bodyweight as maximum acceptable daily intake[10]). After the rats were fasted for 18 h, blood samples were collected from the right atrium of the heart into anticoagulant (3.8 % sodium citrate).

Rat plasma (pseudo) ChE was obtained by centrifuging 1 mL blood at 500 x*g* for 10 min. It was diluted (1 in 200) with 0.1 mol/L phosphate buffer (pH 8.0). Rat erythrocytes (true) AChE was obtained by adding 10 μL red cells to 5 mL saline and centrifuging at 500 x*g* for 10 min. The upper layer was discarded and the remaining red cells were washed (x2) with 5 mL saline. The red cells were resuspended in 0.1 mol/L phosphate buffer (pH 8.0) at a dilution of 1 in 500.[15] Activity was calculated in U/mL for pseudo-ChE and in μmol/min per 10^6 cells for true cholinesterase, as described by Ellman *et al*.[16]

Statistical analysis

Data were analysed using SPSS (version 10) software. One-way analysis of variance (ANOVA, F-test) was used to compare the means of more than two groups, and the significance of differences between the control and each of the different groups was estimated using the LSD value.[19]

Results

In vitro studies

A low concentration of sunset yellow (1.3 x 10^{-4} mol/L) resulted in a decrease in human pseudo- and true ChE activity by 5% and 16%, respectively. When the concentration of sunset yellow was increased, inhibition

reached 50% at 3.3 x 10^{-4} mol/L for pseudo-ChE and 2.4 x 10^{-4} mol/L for true ChE.

A low concentration of sulphanilic acid (7.71 x 10^{-4} mol/L) resulted in a decrease in human pseudo- and true ChE activity by 12.73% and 14.58%, respectively. When the concentration of sulphanilic acid was increased, inhibition reached 50 % at 129 x 10^{-4} mol/L for pseudo-ChE and 122 x 10^{-4} mol/L for true ChE.

A low concentration of carmoisine (8.8 x 10^{-5} mol/L) resulted in a decrease in the human pseudo- and true ChE activity by 17.6% and 31.6%, respectively. When the concentration of carmoisine was increased, inhibition reached 50% at 3.38 x 10^{-4} mol/L for pseudo-ChE and 1.73 x 10^{-4} mol/L for true ChE.

A low concentration of naphthionic acid (2.0 x 10^{-4} mol/L) resulted in a decrease in pseudo-ChE activity by 20%. When the concentration was increased, inhibition reached 50% at 25 x 10^{-4} mol/L. However, naphthionic acid had no effect on true ChE activity *in vitro*.

Double reciprocal curves (Lineweaver-Burke plots of reciprocal velocity 1/v versus reciprocal substrate concentration 1/[s]) for colouring agents at constant concentrations with both human pseudo- and true ChEs gave curves similar to mixed-type (non-competitive and competitive) inhibition for sunset yellow FCF, and similar to non-competitive type inhibition for carmoisine and sulphanilic acid. True ChE inhibition by naphthionic acid was competitive, similar to that discussed by Dixon.[20] The slopes obtained from lines were plotted again against inhibitor concentration [I].[21] The example of human pseudo-ChE inhibition by sulphanilic acid is seen in Figures 1 and 2.

K_i/K_m values for pseudo- and true ChE inhibition by these azo dyes and their by-products indicated that the affinity of sulphanilic acid for both pseudo- and true ChEs was higher than that exhibited by the other compounds. The lowest affinity was seen with sunset yellow FCF, indicating that it produces the greatest inhibition of both pseudo- and true ChEs (Table 1).

The inhibitory effect produced by sunset yellow FCF, carmoisine, sulphanilic acid and naphthionic acid could be abolished by dialysis. However, 90% of original enzyme activity was retrieved in the case of carmoisine and naphthionic acid, while 80% was retrieved in the case of sunset yellow FCF and sulphanilic acid (Table 2).

In vivo studies

The *in vivo* effect of feeding rats on a diet supplemented with sunset yellow FCF, carmoisine, sulphanilic acid or naphthionic acid resulted in a significant decrease ($P<0.05$) in rat pseudo-ChE activity of 23%, 15.1 %, 12.1 % and 18.5 %, respectively. Rat true ChE activity showed significant decreases ($P<0.05$) of 14.5%, 27.8% and 30.6%, respectively (Table 3), for the first three, but naphthionic acid had no effect on activity.

Discussion

Despite the fact that some azo compounds do not normally have cytotoxic, teratogenic or carcinogenic effects, their degraded products (e.g., aromatic amines) may be responsible for a wide range of diseases including asthma, rashes and hyperactivity.[22,23] Romero *et al*.[24] found that superoxide dismutase in the brain of Syrian golden hamsters was reduced by 50 % by the dye Sudan III, leading to a reduction in antioxidant defence. Also, Sudan III promoted reduction in glutathione (GSH)-related activity in brain and liver tissue.[25]

Data from the present study indicated that pseudo- and true ChE inhibition by sunset yellow FCF was of mixed type (competitive and non-competitive), whereas carmoisine and sulphanilic acid produced only non-competitive inhibition. This is similar to the effect on ChE produced by the erythrosine analogues trifluoperzine (TFP) and perphenzine (PPZ), which inhibit true ChE at 20 µmol/L and 40 µmol/L, respectively. This suggests that the phenothazines dye may bind to an allosteric site to produce non-competitive inhibition.[26]

All concentrations of naphthionic acid produced competitive inhibition of pseudo-ChE, where a dramatic increase in enzyme K_p was observed at constant V_{max}. Carmoisine is a potent reversible inhibitor of phenolsulphotransferase P (PSTP), producing 100% inhibition at a concentration of 5 µmol/L. However, it has a less-pronounced effect on phenolsulphotransferase M (PSTM) and monoamineoxidase (MAO) A and B.[27]

The differences in true and pseudo-ChE inhibition observed may be due to conformational changes to the protein;[28] however, variation in the level of true ChE inhibition could be attributed to the effect on the erythrocyte membrane. Acetylcholinesterase in the membrane is a dimer that has identical subunits linked by disulphide bridges that are anchored by a glycoinositol phospholipid at the C terminus of each peptide. An inhibitor that disrupts the disulphide bridges might produce greater conformational change and thus increased inhibition.[29] Previously, this group showed that the azo dyes, their degraded products and dimethyl sulphoxide (DMSO) are anticholinesterase agents,[30] and explained a possible mechanism for the inhibition observed.[15,31]

In vivo study suggests that the differences in enzyme inhibition may be due to different rates of absorption, metabolism and action of the agent used.[28] The results of the present *in vivo* study showed that sunset yellow FCF is the most potent inhibitor of pseudo-ChE, and its by-product sulphanilic acid is the most potent inhibitor for true ChE. This is supported by the work of Goldenring *et al*,[32] who found that long-term daily intraperitoneal injection of sulphanilic acid in young rats produced hyperactivity and induced behavioural change.

The inhibitory effect on ChEs of the dyes and their degraded products reported here reinforces the importance of dye concentration when used as food colour additives. Synthetic coloured food products must be stored away from heat and light, in order to avoid the toxic effects of their by-products. Furthermore, as an alternative to azo dyes, the use of natural colours as food additives is supported. □

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