Synthetic organic hard capsule colouring agents: *in vitro* effect on human true and pseudo-cholinesterases

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ABSTRACT

Hard capsules are made of pure gelatin and small quantities of additives, including colouring agents permitted for use in food. In this study, the effects of three colouring agents (sunset yellow, quinoline yellow and erythrosine) on true and pseudo-cholinesterases (ChE) are assessed in erythrocytes and plasma, respectively. Results indicated that the synthetic compounds affected both true and pseudo ChE activity. The concentration of sunset yellow which caused 50% inhibition (IC_{50}) of true ChE was about 64% that of pseudo-ChE; for erythrosine, IC_{50} was approximately the same for both true and pseudo-ChE; and for quinoline yellow, IC_{50} for true ChE was 25% of pseudo-ChE, although its effect on both true and pseudo-ChE was greater than seen with the other two dyes. Inhibitions of both true and pseudo-ChE were of mixed type (competitive and non-competitive). The enzyme-inhibitor dissociation constant (Ki) indicated that quinoline yellow was most potent and erythrosine was least potent out of the three compounds. Inhibition of both true and pseudo-ChE by each of the three dyes was abolished by dialysis, indicating that the effects were reversible.

KEY WORDS: Cholinesterases. Erythrosine. Capsules. Gelatin. Quinoline yellow. Sunset yellow.

Introduction

Hard capsules used as solid containers to enclose powdered substance are made of pure gelatin (extracted from collagen[1]) and may also contain small quantities of additives that either enable the capsule to be formed more easily or improve its performance in use. One such additive is colouring agent and this has psychological effects on patients.[2]

Colouring agents used in hard capsules are those permitted for use in food, such as synthetic water-soluble dyes, pigments and certain dyes of natural origin,[2] and may be present as mixtures. The synthetic dyes sunset yellow FCF, quinoline yellow and erythrosine are classified chemically as azo, quinophthalone and xanthene dyes, respectively.[3]

Maximum acceptable daily intake (ADI) for sunset yellow FCF is 2.5 mg/kg body weight.[4] It can cause a wide range of health problems, including asthma, rashes, and hyperactivity.[5] Previously, it has been reported that aromatic amines, which are considered to be the main metabolic product of azo dyes, contribute to nervous system dysfunction and chromosomal abnormalities.[6] Moreover, they are considered to be particularly hazardous to mother and child during pregnancy and to those with a history of asthma and chronic infection.[7-9]

Maximum ADI for quinoline yellow is 0.5 mg/kg body weight. It causes allergic responses, severe urticaria, and severe malaise was also noticed in patients treated with naproxen coloured by quinoline yellow.[10]

Maximum ADI for erythrosine is 0.3 mg/kg body weight.[11] It is used widely as a food colouring agent, dramatically and irreversibly alters synaptic transmission at low doses, suggesting that this and other food additives can alter behaviour.

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Cholinesterases (ChEs) are a group of enzymes that degrade choline esters and play a role in neurotransmission in the autonomic and somatic motor nervous systems; however, there have been no systemic investigations of the effects of these compounds on this enzyme group.[12]

This study aims to evaluate the effects of sunset yellow FCF, quinoline yellow and erythrosine on human true and pseudo-ChEs *in vitro*, and some kinetic parameters concerning the interaction between these enzymes and the dyes.

Materials and methods

Reagents

Sunset yellow (Colour Index Number [CI No] 15985, E110), disodium salt of 1-(4 sulphophenylazo)-2-naphthol-6-sulphonic acid; quinoline yellow (CI No 47005, E104), disodium salt of 2-(2,3-dihydro-1,3 dioxo-1H-indene-2-YL)-6,8-quinoline-sulphonic acid; and erythrosine (CI No 45430, E 127), disodium salt of 2,4,5,7 tetraiodofluorescein. All were

Table 1. Values for the enzyme inhibitor dissociation constants of human plasma and erythrocyte ChE inhibited by sunset yellow, quinoline yellow or erythrosine. Results presented as mean ± SEM (n=10)

Inhibitor	Ki x 10^{-6} mol/L		Km x 10^{-4} mol/L		(Ki/Km) x 10^{-2} mol/L		IC50 x 10^{-4} mol/L	
	Plasma	RBCs	Plasma	RBCs	Plasma	RBCs	Plasma	RBCs
Sunset yellow	9.46±0.34$^{\S\Delta*}$	4.26±0.204$^{\Delta\S}$	1.48±0.10**	1.78±0.07	7.11±0.44$^{\S\Delta*}$	2.34±0.11$^{\S\Delta}$	4.23±0.38$^{\S\Delta*}$	2.75±0.28$^{\S\Delta}$
Quinoline yellow	2.36±0.138§**	0.352±0.018$^{\S\bullet}$	1.34±0.07**	1.75±0.05	1.79±0.10*§*	0.199±0.023*§	0.540±0.07*§*	0.136±0.014$^{\S\bullet}$
Erythrosine	126.26±2.77$^{\Delta**}$	60.92±1.34$^{\Delta\bullet}$	1.32±0.06**	1.71±0.11	95.68±0.008$^{\Delta**}$	34.52±0.90$^{\Delta\bullet}$	9.51±0.58$^{*\Delta}$	$^{\Delta}$

K_m: Michaelis constant

Ki: enzyme inhibitor dissociation constant

IC_{50}: concentration required to inhibit 50% of the enzyme activity

* significantly different as compared to corresponding mean value of erythrocyte cholinesterase (P<0.001)

** significantly different as compared to corresponding mean value of erythrocyte cholinesterase (P<0.05)

• significantly different as compared to corresponding mean value using sunset yellow (P<0.001)

Δ significantly different as compared to corresponding mean value using quinoline yellow (P<0.001)

§ significantly different as compared to corresponding mean value using erythrosine (P<0.001)

obtained from Ellis and Everard (England). 5,5` dithiobisnitrobenzoic acid (DTNB) and acetyl thiocholine iodide (AThChI) were obtained from Sigma (St Louis, Mo, USA) and Aldrich (England), respectively. All other chemicals used were of reagent grade.

Subjects

Local ethics committee approval was obtained for the study, which comprised 10 healthy male subjects, free from any clinical symptoms of disease (age range: 25-35 years; red cell count 4.2-5.8 x 10^{12} cell /L; cytocrite value: 40.2-49.9%). The nature of the study was explained to all participating subjects and written informed consent was obtained in each case. Laboratory investigations including complete blood picture, urine analysis, liver and kidney function tests were performed.

Assays

Fresh heparinised blood obtained from each subject in the study was used as a source of both plasma and erythrocyte ChE. Plasma ChE (pseudo-ChE) was obtained by centrifugation of 1 mL heparinised blood at 500 *xg* for 10 min. It was diluted (1 in 200) with phosphate buffer (0.1 mol/L, pH 8.0). Erythrocyte ChE (true ChE) was obtained by adding 10 µL red cells to 5 mL saline, followed by centrifugation at 500 *xg* for 10 min. The upper layer was discarded and the remaining RBCs were washed (x2) with 5 mL saline. The erythrocytes were resuspended in phosphate buffer (0.1 mol/L, pH 8.0) at a dilution of 1 in 500 (7.8 x 10^6 – 9.5 x 10^6 cell/mL).[13]

The effect of each colouring agent on the activity of both plasma and erythrocyte ChE was determined in triplicate at 37°C by the colourimetric method of Ellman *et al*[14] at 412 nm.

Separate assay mixtures contained 2 mL plasma suspension and 2 mL erythrocyte suspension, each in phosphate buffer, together with an amount of enzyme equivalent to 0.08 U/mL, 66 µL DTNB (0.1 mol/L) and 13 µL AThChI (0.5 mmol/L). Control ChE activity was considered as 100% (in the absence of inhibitor) and the enzyme activity after addition of each inhibitor expressed as percentage of control activity.

For determination of inhibition type and the enzyme-inhibitor dissociation constant (K_i) of each colour, the assay was carried out with the inhibitor at a constant concentration and various substrate concentrations (0.5, 1.0, 1.5, and 2.0 mmol/L AThChI). Each colouring agent was added simultaneously to the above mixture in the following concentrations: sunset yellow at 1.3, 2.2, 3.3, and 4.4 x 10^{-4} mol/L, quinoline yellow at 1.35, 2.70, 4.10, 5.50, and 6.80 x 10^{-5} mol/L, and erythrosine at 0.68, 1.00, 1.40 and 2.00 mmol/L. The enzyme-inhibitor mixture and enzyme alone were assayed.[15] Measurement of the concentrations required to inhibit 50% of the enzyme activity (IC_{50}) was considered to reflect the difference in inhibitory power of the three dyes.

Dialysis

Four cellophane tubes each containing 3 mL plasma suspension and quantities of enzymes equivalent to 0.08 unit/mL were used. To each tube only one of the inhibitors was added in the following concentrations: sunset yellow at 3.3 x 10^{-4} mol/L, quinoline yellow at 5.5 x 10^{-5} mol/L or erythrosine at 1 x 10^{-3} mol/L. Controls were prepared without addition of inhibitor.

The tubes dialysed each separately overnight against phosphate buffer (0.1 mol/L, pH 8.0) at 4°C with occasional changes of the buffer. All the dialysed tubes were assayed for ChE activity as described before and compared with undialysed enzyme with inhibitor.

Results presented in Table 1 and Figures 1, 2 and 3 were evaluated statistically using two-way analysis of variance. Paired *t*-test was used for comparison of results before and after dialysis.[16]

Results

At low sunset yellow concentration (1.3 x 10^{-4} mol/L) the activities of pseudo and true ChE decreased by 5% and 16%, respectively. Increasing the sunset yellow concentration to

Table 2. Effect of dialysis on plasma ChE activity before and after the addition of sunset yellow, quinoline yellow or erythrosine

Inhibitor	[I] mol/L	% ChE (activity)	
		Before dialysis	After dialysis
Sunset yellow	3.3×10^{-4}	56.75 ± 1.05	83.83 ± 1.10*
Quinoline yellow	5.5×10^{-5}	52.63 ± 0.90	79.85 ± 1.08*
Erythrosine	1×10^{-3}	49.86 ± 0.94	91.22 ± 9.56*

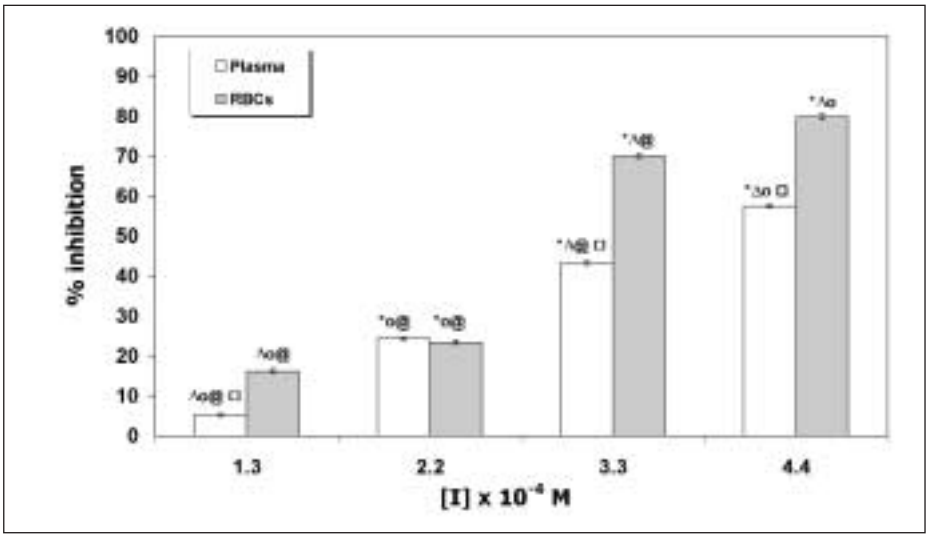
Results presented as mean ± SEM (n=10)

Mean actual activity of plasma ChE ± SEM was 1.88 ± 0.05 μmol/min/mL

Control was considered as 100% activity

* significantly different as compared to corresponding values before dialysis (P<0.001)

Fig 1. Inhibition percentages of plasma and erythrocyte cholinesterases by sunset yellow.



Results expressed as mean ± SEM for 10 individuals

The actual mean activity of plasma and erythrocyte cholinesterases in the absence of inhibitor (sunset yellow) are 1.88±0.05 μmol/min/mL and 1.2±0.02 μmol/min/10^9 cells respectively (represent 100% activity or 0% inhibition)

□ Indicates significant difference compared to corresponding inhibition percentage of erythrocyte cholinesterase at the same concentration of sunset yellow (P < 0.001).

* significant difference compared to corresponding percentages using 1.3×10^{-4} mol/L (P<0.001)

Δ significant difference compared to corresponding percentages using 2.2×10^{-4} mol/L (P<0.001)

o significant difference compared to corresponding percentages using 3.3×10^{-4} mol/L (P<0.001)

@ significant difference compared to corresponding percentages using 4.4×10^{-4} mol/L (P<0.001)

3.3×10^{-4} mol/L resulted in inhibition of 43% and 70%, respectively (Figure 1).

Low concentration of quinoline yellow (1.35×10^{-5} mol/L) resulted in a 2.6% decrease in the activity of pseudo-ChE, while activity of true ChE decreased to 48% of normal. With increasing concentrations of quinoline yellow up to 6.8×10^{-5} mol/L, inhibition of pseudo-ChE and true ChE activities reached 53% and 87% of normal, respectively (Figure 2).

At a low concentration of erythrosine (0.68 mmol/L) there was a decrease in plasma and erythrocyte ChE activities, which reached 24% and 38% of normal, respectively. With increased concentrations of erythrosine up to 1.4 mmol/L, the inhibition of pseudo-ChE and true ChE activities reached 52% and 64% of normal, respectively (Figure 3).

Double reciprocal curves (Lineweaver-Burke plots of reciprocal velocity 1/v versus reciprocal substrate concentration 1/[S]) for sunset yellow, quinoline yellow and erythrosine (at constant concentration) with both pseudo- and true ChE gave curves similar to those mentioned by Steinberg and Cramer[17] for mixed-type inhibition (i.e. inhibition has some of the characteristics of the competitive and some of the non-competitive mechanisms), taking human erythrocyte ChE inhibited by quinoline yellow as an example (Figure 4a). The slopes obtained from the lines were replotted versus inhibitor concentration [I] (Figure 4b) according to Cleland.[18]

Derived IC_{50} and Ki (enzyme-inhibitor dissociation constant) indicated that quinoline yellow had higher inhibitory power on both plasma and erythrocyte ChE than

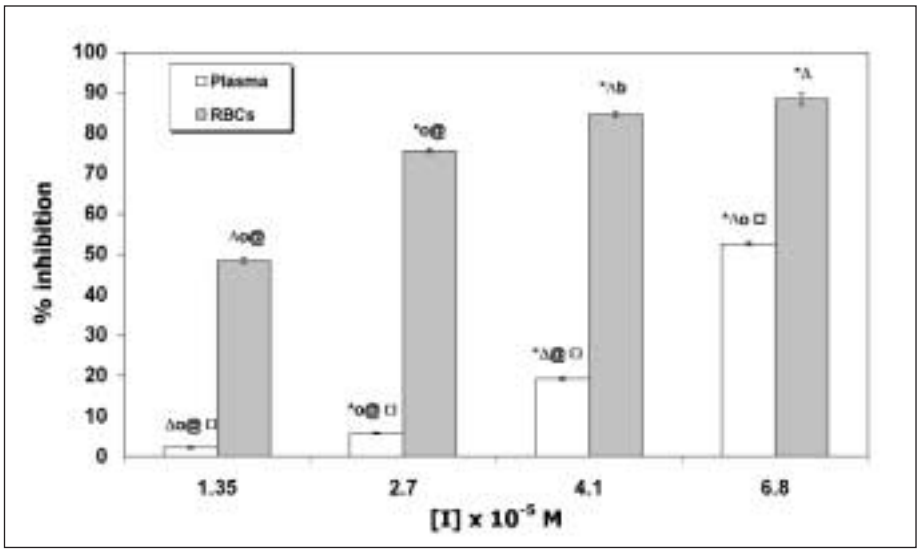


Fig. 2. Inhibition percentages of plasma and erythrocyte cholinesterases by quinoline yellow.

Results expressed as mean ± SEM for 10 individuals
The actual mean activity of plasma and erythrocyte cholinesterases in the absence of inhibitor (quinoline yellow) are 1.88±0.05 μmol/min/mL and 1.2±0.02 μmol/min/10^9 cells respectively (represent 100% activity or 0% inhibition)

□ significant difference compared to corresponding inhibition percentage of erythrocyte cholinesterase at the same concentration of quinoline yellow ($P<0.001$).
* significant difference compared to corresponding percentages using 1.35 x 10^{-5} mol/L ($P<0.001$)
Δ significant difference compared to corresponding percentages using 2. 7x 10^{-5} mol/L ($P<0.001$)
o significant difference compared to corresponding percentages using 4.1 x 10^{-5} mol/L ($P<0.001$)
@ significant difference compared to corresponding percentages using 6.8 x 10^{-5} mol/L ($P<0.001$)
b significant difference compared to corresponding percentage of erythrocyte cholinesterase with 6.8 x 10^{-5} mol/L quinoline yellow ($P < 0.01$).

did sunset yellow or erythrosine (Table 1).

Inhibitory effects of sunset yellow, quinoline yellow and erythrosine could be abolished by dialysis, recovering about 90% of their original activity. This indicated that the three dyes are reversible inhibitors (Table 2).

Discussion

The results obtained *in vitro* by addition of different concentrations of sunset yellow, quinoline yellow or erythrosine at constant substrate and enzyme concentrations, indicate that each dye has a different inhibitory power. However, dose-dependent inhibition of true and pseudo-ChE activities demonstrates that these dyes are strong inhibitors.

Water-soluble dyes such as sunset yellow are biotransformed by intestinal microorganisms in the gastrointestinal tract, and their toxicity, mutagenicity and carcinogenicity in the intestine or liver may be attributed to their metabolites.[19]

Decolourisation of azo dyes can involve both adsorption to cell biomass and degradation by azo-bond reduction during anaerobic digestion. Degradation is expected to form toxic aromatic amines.[8] The kinetics of azoreductase, responsible for decolourisation of structurally simple azo dyes, suggests a competitive inhibition model.[20]

With regard to IC_{50} values of the three dyes against human erythrocytes, quinoline yellow was found to have the highest potency ($P<0.001$), being 20 and 60 times more potent than sunset yellow and erythrosine, respectively. Indeed, the IC_{50} for quinoline yellow was comparable with values for the organophosphorus compounds dyfonateoxon (2.15 x 10^{-5} mol/L), paraoxon (1.99 x 10^{-5} mol/L) and malaoxon (7.04 x 10^{-5} mol/L) in bovine erythrocytes.[21]

Previous studies indicated that erythrosine and related dyes inhibit membrane transport of dopamine by increasing potassium permeability of neurons,[22] thereby decreasing dopamine uptake *in vitro*.[23]

Yu and associates[24] reported that lesions of apparent ischaemic origin in rat sciatic nerve were induced photochemically by laser irradiation combined with systemic administration of the photosensitising dye erythrosine. At the irradiated nerve site, occlusion of blood vessels with aggregated thrombocytes, fibrins and deformed erythrocytes was seen, supporting the theory that a photochemical reaction caused intraneural ischaemia.

From the kinetic studies of AThChI hydrolysis at constant enzyme and substrate concentrations in the presence of different dye concentrations, it was possible to determine the type of inhibition and enzyme inhibitor dissociation constants. The results obtained indicated that inhibition of both plasma and erythrocyte ChEs in the presence of these three colouring agents was of mixed (competitive and non-competitive) type, which is similar to the inhibition of ChE produced by the erythrosine analogues trifluoperazine and perphenazine. These two compounds showed that inhibition of erythrocyte ChE is of mixed type at concentrations above 20 μmol/L and 40 μmol/L, respectively.[25]

Ki/Km values for plasma and erythrocyte ChE inhibition by these dyes indicated that the affinity of erythrosine for both true and pseudo-ChE was higher than for the other two dyes studied. Ki values for true and pseudo-ChEs

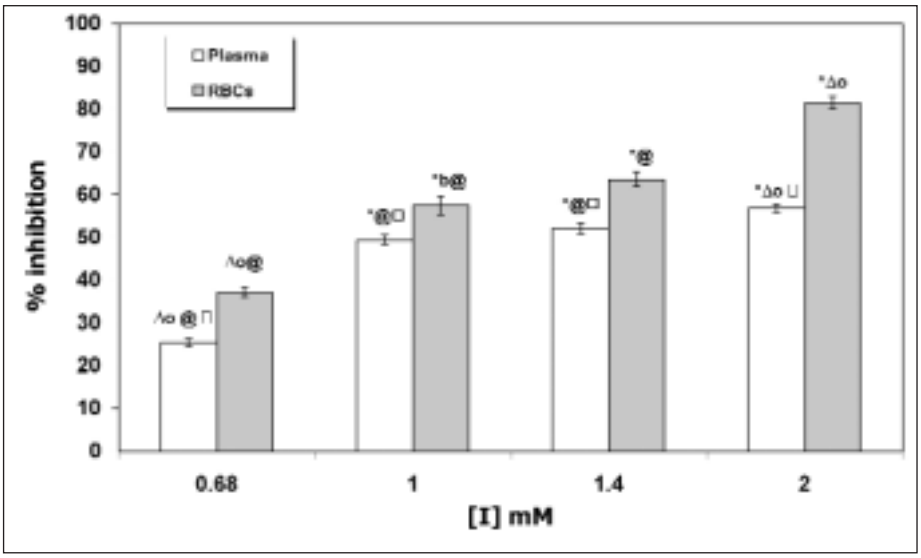


Fig. 3. Inhibition percentages of plasma and erythrocyte cholinesterases by erythrosine.

Results expressed as mean ± SEM for 10 individuals
The actual mean activity of plasma and erythrocyte cholinesterases in the absence of inhibitor (Erythrosine) are 1.88±0.05 μmol/min/mL and 1.2±0.02 μmol/min/10^9 cells respectively (represent 100% activity or 0% inhibition)

□ significant difference compared to corresponding inhibition percentage of erythrocyte cholinesterase at the same concentration of erythrosine ($P<0.001$).
* significant difference compared to corresponding percentages using 0.68 mmol/L ($P<0.001$).
Δ significant difference compared to corresponding percentages using 1 mmol/L ($P<0.001$)
o significant difference compared to corresponding percentages using 1.4 mmol/L ($P<0.001$)
@ significant difference compared to corresponding percentages using 2 mmol/L ($P < 0.001$)
b significant difference compared to corresponding inhibition percentage of erythrocyte cholinesterase with 1.4 mmol/L ($P < 0.05$)

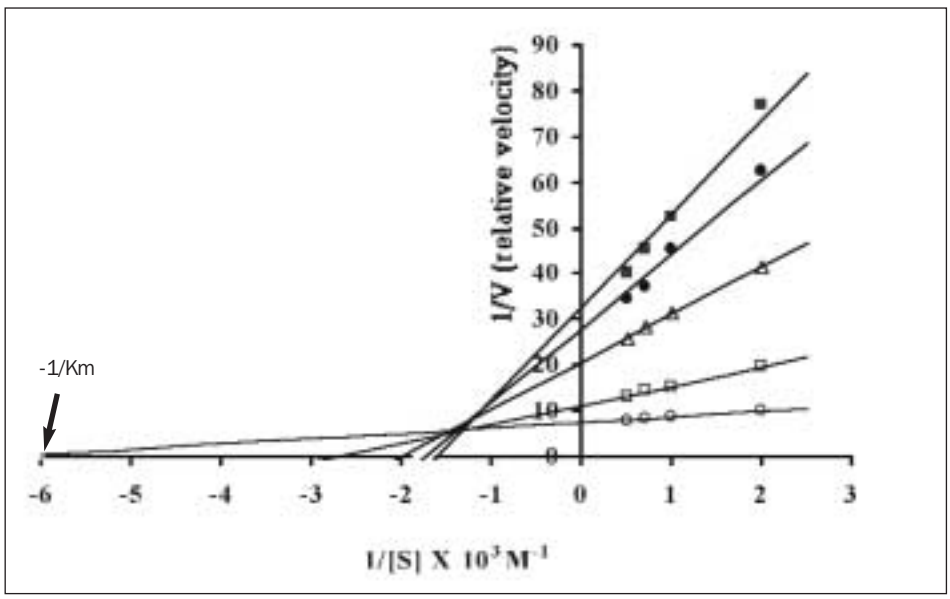


Fig.4a. Lineweaver-Burk plot of human RBC AChE inhibited by quinoline yellow: (o) control, (□) 1.35 x 10^{-5} mol/L, (△) 2.70 x 10^{-5} mol/L, (●) 4.10 x 10^{-5} mol/L. (■) 5.5 x 10^{-5} mol/L
Values are means ± SEM for 10 individuals

indicated that quinoline yellow potency was greatest, followed by sunset yellow and then erythrosine.

Difference in degree of inhibition of erythrocyte ChE could be due to the behaviour of each of the inhibitor towards the erythrocyte membrane. ChE in erythrocyte membranes is a dimer where identical subunits are linked by a disulphide bridge and anchored in the membrane by a glycoinositol phospholipid at the C terminus of each peptide. Thus, disruption of the disulphide bridges may allow greater changes in the conformation of the enzyme, and might be the basis of the higher degree of inhibition seen.[26]

Reversibility of dye inhibition was confirmed by dialysis, which confirmed the results of previous study.[27]

From the results obtained in the present study, it is concluded that the differences in the rate of inhibition of both plasma and erythrocyte ChE by sunset yellow, quinoline yellow or erythrosine may be attributed to the variation in dye composition and to the difference in the mode of interaction of each with the active site of the enzyme. Moreover, the abolition of dye-induced inhibition

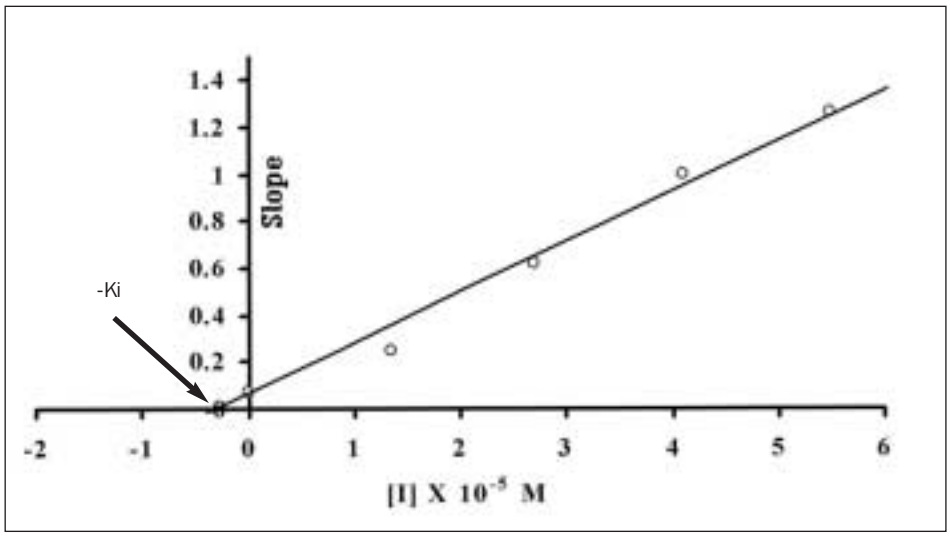


Fig.4b. Cleland replot of the slopes obtained from Figure 4a against the inhibitor concentration [I]

of plasma and erythrocyte ChEs by dialysis indicates that these dyes are reversible inhibitors.

Our results support the use of natural colours instead of artificial dyes in food additives or in the manufacture of hard capsules. □

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