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THE HYDROLYSIS OF SUCCINYLDITHIOCHOLINE AND RELATED THIOCHOLINE ESTERS BY HUMAN PLASMA AND PURIFIED CHOLINESTERASE

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Abstract—The kinetic properties of cholinesterase (ChE) present in plasma were compared with those of purified human ChE using the substrates succinyldithiocholine (SDTCh), acetylthiocholine (AcTCh) and butyrylthiocholine (BuTCh). SDTCh was hydrolysed at two sites; a site with a low K_m (K_{m1} 11.4 ± 3.3 μM) with a V_{max} of 0.06 μmol/min/ml and a site with a high K_m (K_{m2} 132.4 ± 14.8 μM) and a V_{max} of 0.107 μmol/min/ml. The K_{m2} site was absent in the sample of purified ChE. The related thiocholine esters, AcTCh and BuTCh were hydrolysed at two sites by both plasma and purified ChE. This indicated that the K_{m2} site which hydrolysed SDTCh was not ChE. The identity of this component in plasma remains unknown but it was shown not to be albumin. The anticholinesterase agents soman and pyridostigmine were used to demonstrate the direct relationship between inhibition of plasma ChE and hydrolysis of SDTCh at the low concentrations present clinically (20 μM). Whereas high concentrations of SDTCh (200 μm) could be partly hydrolysed by an enzyme present in plasma which is insensitive to ChE inhibitors. In a limited study on the plasma from two "atypical" individuals (Dibucaine number < 20) all three substrates were hydrolysed at a single site with a higher K_m than the K_{m2} site present in normal plasma. The clinical implications of these results are discussed.

Plasma cholinesterase (ChE acylcholine acyl-hydrolase EC 3.1.1.8) exists as a series of multiple molecular forms which can be classified as isoenzymes by virtue of their substrate specificity, inhibitor susceptibility and interconvertibility [1]. ChEs are distinguished from other esterases by a high affinity for choline esters and their thiol analogues [2]. Kinetic studies on plasma and semi-purified preparations of ChE have demonstrated that the hydrolysis of choline esters does not follow normal Michaelis–Menten kinetics [3–7]. In addition a number of genetic variants of the enzyme have been identified as "unusual" by using inhibitors such as dibucaine [8] and fluoride [9]. The discovery of these variants arose from a study of individuals who were unusually sensitive to the muscle relaxant succinyldicholine (SDCh).

SDCh is a neuromuscular blocking drug used for endotracheal intubation, endoscopies and electroconvulsive therapy. Its short duration of action (3–5 min) is due to its rapid hydrolysis by serum ChE in normal individuals [10]. SDCh sensitivity has been reported in approximately 0.003% of the population and results in a prolonged apnoea after SDCh administration. The apnoea can be reversed by the administration of a commercially available lyophilised human serum ChE preparation [11]. Kinetic studies on the hydrolysis of SDCh by the plasma of normal and "atypical" individuals have demonstrated that there was a large increase in the K_m of the enzyme in "atypical" individuals for SDCh [12, 13].

The main objective of the present study was to investigate the kinetic properties of the enzyme present in normal plasma which is responsible for the hydrolysis of SDCh at the concentrations used clinically. The kinetic properties of normal male and female plasma ChE were compared with purified human ChE using the substrates acetylthiocholine (AcTCh), butyrylthiocholine (BuTCh) and succinyldithiocholine (SDTCh). The thiol analogues of the choline esters were used so that a direct comparison could be made of the Michaelis constants K_m and V_{max} using the colorimetric method of Ellman *et al.* [14] for the three substrates over a wide range of substrate concentrations. Plasma was routinely used in this study since it has been shown to have similar properties to serum [15]. The ChE inhibitors pyridostigmine and soman were then used to investigate the relationship between ChE activity and SDTCh hydrolysis in normal plasma and purified ChE. A limited study on the plasma from two "atypical" individuals was also included.

MATERIALS AND METHODS

Blood samples were taken by venepuncture from healthy male and female volunteers aged 19–28 years and placed into tubes containing EDTA. Samples were spun for 2 min in an Eppendorf centrifuge at 9980 *g* and the plasma removed for ChE assay as described below. Samples were assayed fresh or stored at −20°. All female volunteers were taking an oral contraceptive.

Human cholinesterase (Serum-Cholinesterase, Behringwerks AG, Marburg, Hoechst Pharma AG Zurich) purified 10,000-fold by the method of Haupt *et al.* [16] and equivalent to 500 ml normal plasma

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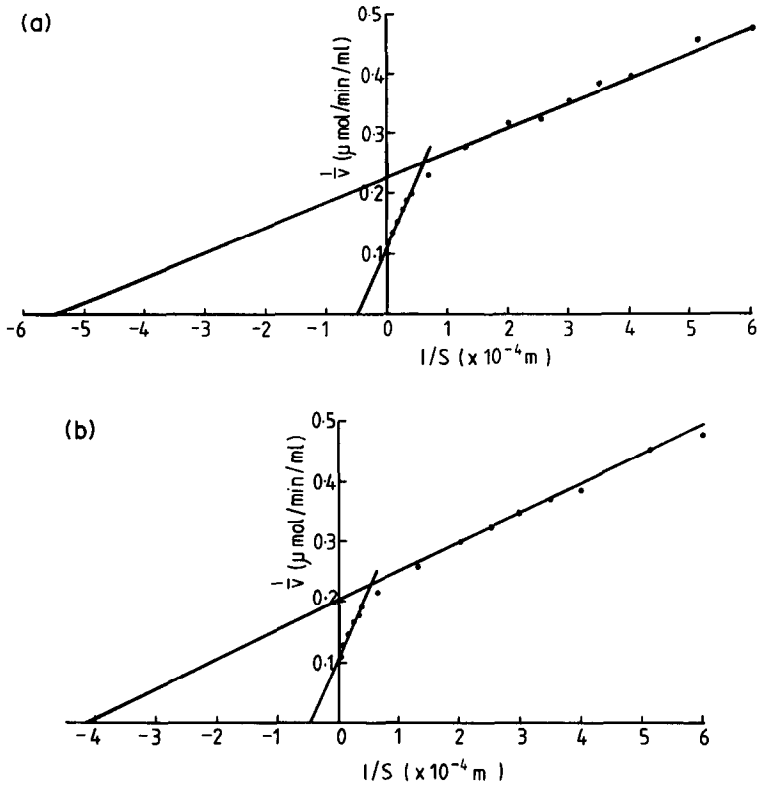

Fig. 1. A Lineweaver–Burk plot showing the effect of substrate concentration on the hydrolysis rate of butyrylthiocholine by (a) normal plasma from an individual male, (b) purified cholinesterase.

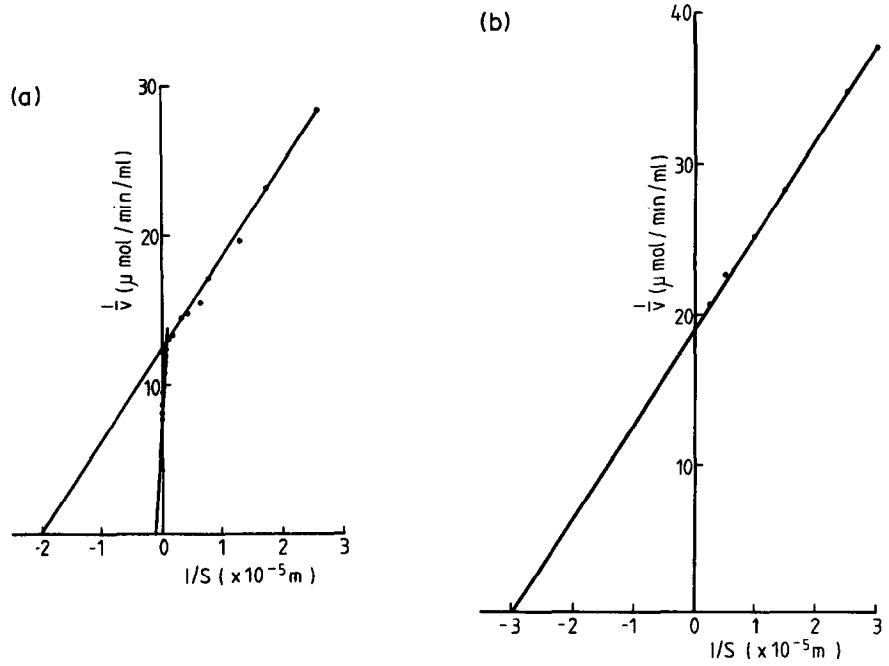

Fig. 2. A Lineweaver–Burk plot showing the effect of substrate concentration on the hydrolysis rate of succinyldithiocholine by (a) normal plasma from an individual male, (b) purified cholinesterase.

Table 1. Kinetic parameters for the hydrolysis of acetylthiocholine, butyrylthiocholine and succinyldithiocholine by human plasma, purified cholinesterase and albumin (The results are expressed as the mean ± S.E.M.)

Substrate	Enzyme source	Sex	N	K_{m1} (μM)	V_{max1} (μmol/min/ml)	K_{m2} (μM)	V_{max2} (μmol/min/ml)
AcTCH	Plasma	♀	5	50.2 ± 2.6	1.8 ± 0.08*	346.6 ± 19.7	3.06 ± 0.13*
	Plasma	♂	6	45 ± 3.9	2.4 ± 0.14	317 ± 20.7	3.95 ± 0.25
	Purified ChE	—	3	43.3 ± 5.5	2.6 ± 0.32	296 ± 27.9	3.97 ± 0.34
	Atypical plasma	♀	1	—	—	928	0.65
	Atypical plasma	♂	1	—	—	999	1.47
BuTCh	Plasma	♀	5	21 ± 1.9	2.7 ± 0.16†	226 ± 6.7‡	5.2 ± 0.21†
	Plasma	♂	6	22 ± 1.6	3.8 ± 0.28	189 ± 7.4‖	7.01 ± 0.47
	Purified ChE	—	3	17.7 ± 4.5	4.4 ± 0.75§	243 ± 19.1	9.9 ± 0.99§
	Atypical plasma	♀	1	—	—	249	1.28
	Atypical plasma	♂	1	—	—	302	3.29
SDTCh	Plasma	♀	5	32.6 ± 8.7¶	0.047 ± 0.005	191.2 ± 6.3¶	0.088 ± 0.004
	Plasma	♂	7	11.4 ± 3.3	0.060 ± 0.005	132.4 ± 14.8	0.107 ± 0.008
	Purified ChE	—	3	3.4 ± 0.23	0.056 ± 0.002	—	—
	Atypical plasma	♀	1	—	—	319	0.082
	Atypical plasma	♂	1	—	—	522	0.162
	Albumin (4 mg/ml)	—	1	—	—	385	0.008
	Albumin (10 mg/ml)	—	1	—	—	417	0.01

* ♀ < ♂ ($P < 0.016$). † ♀ < ♂ ($P < 0.01$). ‡ ♀ > ♂ ($P = 0.01$). § ChE > ♂ ($P < 0.016$). ‖ ♂ < ChE ($P = 0.013$). ¶ ♀ < ♂ ($P < 0.026$).

was diluted 1/500 with the appropriate buffer and then diluted further as for plasma.

ChE assay. All samples were assayed using the method of Ellman *et al.* [14]. AcTCh, BuTCh and 5,5 dithio-bis(2-nitrobenzoic acid) (DTNB) were purchased from BDH Ltd. SDTCh was synthesized at CDE Porton Down according to the method of Goodyer and Mautner [17].

Plasma samples were diluted 1/400 and 1/500 with 0.1 M pH 8.0 sodium phosphate buffer before assay with AcTCh (1.7×10^{-5} M–1.2×10^{-2} M) and BuTCh (4.5×10^{-6} M–2×10^{-2} M) respectively. The hydrolysis of SDTCh (3.9×10^{-6}–1.0×10^{-3} M) was measured in 0.05 M Tris–hydrochloride buffer pH 7.4. The plasma was diluted 1/10 with the same buffer before assay. Normal plasma has the same enzyme activity in 0.05 M Tris or phosphate buffer [18].

Absorbance changes were measured using a Pye Unicam PU 8800 UV/VIS spectrophotometer at 412 nm and 30°. Samples were measured for up to 5 min. The reaction was linear during this time period. Data were corrected for spontaneous hydrolysis of the substrate.

Hydrolysis of SDTCh by human serum albumin. Human albumin (Sigma) was made up in 75 mM Tris–hydrochloride buffer pH 7.4 at 4.0 and 10.0 g/100 ml and assayed as for human plasma with the substrate SDTCh (1.7×10^{-4} M–1×10^{-3} M).

Dibucaine number and protein determination. Dibucaine number was determined by the method of Kalow and Genest [8]. Plasma protein concentration was determined by the Biuret reaction [19] on an Abott ABA using A-gent reagents.

K_m *and* V_{max} *determination.* The data was initially plotted using the double reciprocal plot of Lineweaver and Burk [20]. The two lines obtained with each substrate were analysed separately by the direct linear plot of Eisenthal and Cornish-Bowden [21, 22]. The Michaelis constants K_m and V_{max} were calculated from this plot.

Effect of inhibitors. Pyridostigmine bromide and soman (1,2 2-trimethyl propyl methyl phosphonofluoridate) > 90% pure by the colorimetric method of Marsh and Neal [23] were synthesized at CDE.

Plasma or ChE was incubated at 37° with pyridostigmine (90 min) or soman (10 min), i.e. the time required to reach maximum inhibition for each concentration tested. The samples were then diluted with the appropriate buffer for assay of BuTCh (10 mM) and SDTCh (20 and 200 μm) hydrolysis as previously described.

RESULTS

The hydrolysis of AcTCh, BuTCh and SDTCh

1. Normal plasma and purified ChE. A comparison of the effect of substrate concentration on the hydrolysis rate of BuTCh and SDTCh is shown in Figs 1 and 2. A Lineweaver–Burk plot of the hydrolysis of BuTCh by normal plasma from an individual male (Fig. 1a) and purified ChE (Fig. 1b) was clearly biphasic. Similar results were obtained using AcTCh

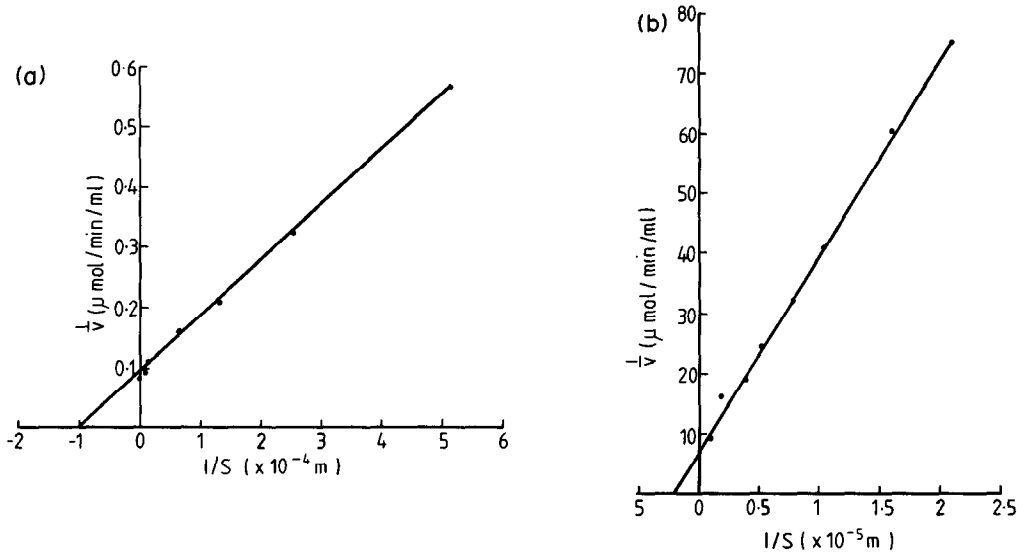

Fig. 3. A Lineweaver–Burk plot showing the effect of substrate concentration on the hydrolysis rate of (a) butyrylthiocholine by "atypical" plasma (female), (b) succinyldithiocholine by "atypical" plasma (male).

as the substrate. In comparison a Lineweaver–Burk plot of the hydrolysis of SDTCh was biphasic in the normal plasma from another individual male (Fig. 2a) but linear in purified ChE (Fig. 2b). Increasing the concentration of SDTCh to 1×10^{-3} M with purified ChE did not increase the reaction rate above that measured at 5×10^{-6} M SDTCh. An analysis of the K_m and V_{max} values obtained from the slopes of the two lines (Table 1) clearly demonstrated that the enzyme present in normal male and female plasma hydrolysed AcTCh, BuTCh and SDTCh at two different sites; a site with a low K_m (K_{m1}) and a site with a high K_m (K_{m2}). There was also a significant difference in the corresponding V_{max1} and V_{max2} values for the three substrates studied.

The hydrolysis of both AcTCh and BuTCh by purified ChE produced values for the two K_m and V_{max} values which were of the same order of magnitude as those in plasma. In comparison SDTCh was hydrolysed by purified ChE at a single site with a low K_m (Table 1). This indicated that the site with the high K_m was lost during purification. A comparison of the Michaelis constants obtained for the three substrates for normal plasma and purified ChE showed a general order for K_m of SDTCh < BuTCh ≪ AcTCh whereas the V_{max} was in the order BuTCh > AcTCh ≫ SDTCh. There were statistically significant differences in the kinetic values obtained for male and female plasma and purified ChE, which are summarized in Table 1.

2. *"Atypical" plasma.* The effect of substrate concentration on the hydrolysis of BuTCh and SDTCh is shown in Fig. 3. The rates of hydrolysis of both substrates were linear over the concentrations used. A similar result was obtained using AcTCh as the substrate. A comparison of the kinetic constants obtained in the single male and female individuals studied showed that all three substrates were hydrolysed at a single site with a higher K_m than the K_{m2} site present in normal plasma and purified ChE (Table 1). The V_{max} was reduced by at least 50% for AcTCh and BuTCh but there was no significant difference in the maximum hydrolysis rates of SDTCh when compared to normal plasma. The ratio of K_{m2} "atypical"/typical for male plasma was 3.95 for SDTCh, 3.2 for AcTCh and 1.6 for BuTCh. A further interesting observation was that albumin had a similar K_m for SDTCh as "atypical" plasma but was only hydrolysed at about 10% of its rate (Table 1).

Dibucaine numbers and plasma protein determinations

The dibucaine numbers for normal plasma were 77.6 ± 1.1 (male) and 80.9 ± 1.2 (female). The values obtained for "atypical" plasma were 13.3 (male) and 13.5 (female). The plasma protein concentration of all samples was within the normal range (5.7–7.9 g/100 ml).

The effect of ChE inhibitors on the hydrolysis of BuTCh and SDTCh by normal plasma and purified ChE

The effect of soman concentration on the hydrolysis rate of 10 mM BuTCh and 20 and 200 μM SDTCh by normal plasma is shown in Fig. 4(a). The two concentrations of SDTCh were chosen to measure the hydrolysis at the K_{m1} and K_{m2} sites. The plasma ChE activity (as measured by the hydrolysis of BuTCh) was completely inhibited by 3.0×10^{-7} M soman. At the same concentration the hydrolysis of 20 μM SDTCh was inhibited by 90% whereas the hydrolysis of 200 μM SDTCh was inhibited by 58%. Increasing the soman concentration by one order of magnitude produced the same results. The % inhibition of BuTCh hydrolysis was significantly higher ($P < 0.05$) than those obtained with SDTCh at all soman concentrations except at 6.0×10^{-8} for 20 μM SDTCh and 1.0×10^{-8} M for 200 μM SDTCh.

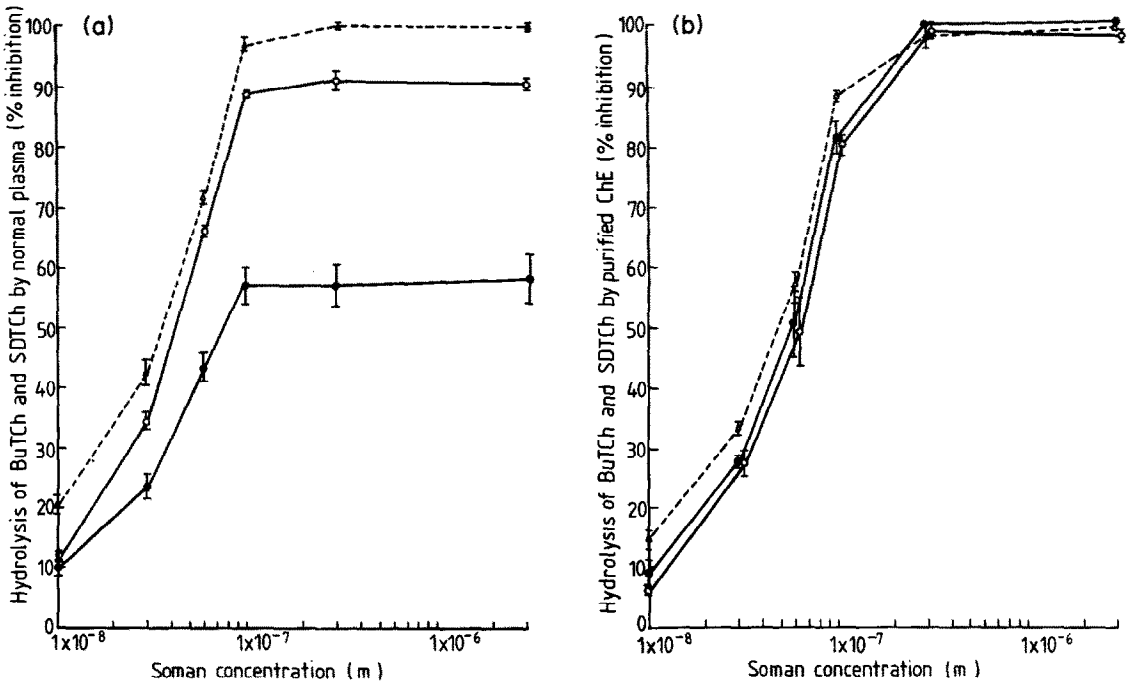

Fig. 4. The effect of soman concentration on the hydrolysis rates of butyrylthiocholine 10 mM (Δ . . . Δ), succinyldithiocholine 20 μM (○——○) and succinyldithiocholine 200 μM (●——●) by (a) normal plasma, (b) purified cholinesterase. Each point represents the mean ± S.E.M. of 3 experiments.

Similar results were produced for pyridostigmine except that a higher concentration (1.0×10^{-4}) was required to produce 98% inhibition of BuTCh hydrolysis. The corresponding values for the inhibition of 20 and 200 μM SDTCh hydrolysis were 88.0 and 58.0% respectively. A single experiment on "atypical" plasma inhibited by soman and assayed with 200 μM SDTCh and 10 mM BuTCh produced the same dose/response curves as normal plasma.

In comparison the dose/response inhibition of purified ChE by soman as measured by the hydrolysis rate of BuTCh and 20 and 200 μM SDTCh produced almost identical sigmoid curves for both substrates (Fig. 4(b)). The inhibition of 20 μM SDTCh hydrolysis was only significantly lower than BuTCh at soman concentrations of 1.0×10^{-7} and 1.0×10^{-8} M. The concentration of soman required to produce complete inhibition of the purified ChE preparation was the same as for plasma (3.0×10^{-7} M). Similar results were produced with pyridostigmine except that a concentration of 1.0×10^{-4} M was required to produce complete inhibition of the enzyme.

DISCUSSION

The present study has shown that the hydrolysis of SDTCh by human plasma did not follow normal Michaelis–Menten kinetics. The substrate was hydrolysed at two sites; one with a low K_m and V_{max} and the other with a high K_m and V_{max}, whereas purified ChE hydrolysed SDTCh at a single site with a low K_m. This contradicts the findings of Hersh *et al.* [13] who reported a single value of 0.032 mM for the K_m for SDTCh hydrolysis by serum ChE. A similar K_m value of 0.027 ± 0.006 mM could be obtained with the present data by computing the line of best fit and ignoring the large deviations at each end of the line. Kalow [12] also reported that the hydrolysis of SDCh by normal plasma produced complicated reaction kinetics but did not quote any kinetic values.

The clinical dose of SDCh for adults is 20–100 mg i.v. which is estimated to produce an initial plasma concentration of 16–80 μM. This concentration would be hydrolysed by the K_{m1} site of ChE shown to be present in normal plasma. A comparison of the kinetic constants (K_{m1} and V_{max1}) obtained for purified ChE and normal plasma confirmed that it was this site which is responsible for the hydrolysis of SDTCh at the concentrations used clinically. The K_{m2} site was either removed during the purification of ChE indicating the presence of other esterases in normal plasma which could hydrolyse SDTCh non-specifically at high concentrations, or the ChE molecule may have been modified. Since the apnoea produced by SDCh in "atypical" individuals can be reversed by i.v. administration of the purified serum-ChE used in this study [11] this indicates that the K_{m2} site may only play some role in the hydrolysis of the high concentrations of SDCh which are present initially.

The effect of the ChE inhibitors soman and pyridostigmine on the hydrolysis of SDTCh by plasma and purified ChE demonstrated two important

points. First, that the low concentrations of SDTCh used clinically are hydrolysed by ChE present in plasma. This confirms observations that the neuromuscular effects of SDCh are prolonged and potentiated following administration of anticholinesterase drugs in man [24]. Second, that high concentrations of SDTCh can be hydrolysed, to a limited extent, by an enzyme present in plasma with a high K_m which is insensitive to ChE inhibitors. This enzyme is not a carboxylesterase, because they are sensitive to ChE inhibitors [25], or an arylesterase, because they do not hydrolyse thiol analogues of choline esters [26]. One possibility was that this component was albumin since human plasma albumin and gamma globulin bind SDCh both *in vivo* and *in vitro* [27]. The ability of SDTCh to bind to albumin was confirmed but the hydrolysis rate was an order of magnitude too low to account for its hydrolysis in the presence of ChE inhibitors. The identity of this component in plasma remains unknown.

In contrast the hydrolysis of BuTCh and AcTCh by plasma and purified ChE produced similar results. The effect of substrate concentration on the hydrolysis of BuTCh and AcTCh by plasma and purified ChE confirmed previous observations that ChE has two active sites [3–7]. This has been interpreted as either showing the presence of two enzymes [3, 4] or as enzyme activation at high substrate concentrations [5] or as the presence of a modifier or an allosteric site [7]. In a recent review Main [28] suggested that the substrate reactions may involve cooperative effects or that the enzymes may exist in active and inactive forms that are interchangeable. However, a complete kinetic analysis will not be possible until each of the active enzyme species has been separated and purified.

The K_m values for the three substrates hydrolysed by plasma and purified ChE were in the order SDTCh < BuTCh ≪ AcTCh and demonstrated the importance of the hydrophobic area in the binding of the choline moiety at the anionic site (reviewed by Brown *et al.* [29]). Whereas the catalytic activity at the esteratic site was in the expected order of BuTCh > AcTCh ≫ SDTCh [29]. In addition, the significantly lower values ($P < 0.016$) obtained in females for the V_{max1} and V_{max2} values for AcTCh and BuTCh confirm the study by Sidell and Kaminski [30] that plasma ChE activity is higher in men than women up to the age of 60 years. All the female subjects used in this study were taking an oral contraceptive which is also known to produce a fall in serum ChE and a change in serum proteins [31].

The numerous studies on "atypical" serum ChE have concluded that it is a structurally modified enzyme which is less negatively charged at the anionic site [32, 33]. In the present study the kinetic constants obtained for the hydrolysis of AcTCh, BuTCh and SDTCh by "atypical" plasma confirmed previous observations of a decreased apparent affinity for each of these substrates [12, 13] and a decreased maximal hydrolysis for AcTCh and BuTCh [12] but not for SDTCh [13] when compared with normal plasma. One interesting feature of the comparison was that the enzyme present in "atypical" plasma had a higher K_m for all three substrates than the K_{m2} site present in normal plasma. This demonstrated that the difference between "atypical" and normal plasma was not just the absence of the K_{m1} site but confirms that it is a structural modification of the active site.

One aspect of the present study was that it had to be assumed that the thiol analogues of the substrates used would have similar kinetic properties to their parent oxygen analogues. It has been demonstrated that the hydrolysis of AcTCh and BuTCh do not follow normal Michaelis–Menten kinetics like their parent oxygen analogues [34], but it remains to be confirmed that SDCh behaves like SDTCh. However, SDTCh has been used in a study of serum from individuals showing hypersensitivity to suxamethonium [35]. Also, the contribution of the hydrolysis of succinyl monothiocholine (SMTCh) was not evaluated in the present study but it has been reported [36] that serum ChE has a very low K_m (8.4×10^{-3}) for SMTCh and therefore the contribution of SMTCh to the hydrolysis of SDTCh should be negligible.

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