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THE INHIBITION BY D-TUBOCURARINE OF HORSE SERUM CHOLINESTERASE-CATALYZED HYDROLYSIS OF BUTYRYLCHOLINE AND BENZOYLCHOLINE

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Abstract—Experiments with butyrylcholine and benzoylcholine as substrates revealed that inhibition of horse serum cholinesterase by D-tubocurarine is linearly mixed. The ratio between K_i and K_i' is practically the same for both substrates, K_i' being five times as large as K_i. The difference is in the degree of inhibition: D-tubocurarine is a more potent inhibitor of butyrylcholine than benzoylcholine hydrolysis. The mixed inhibition of serum cholinesterase by D-tubocurarine can be explained by the allosteric binding of D-tubocurarine to the enzyme protein and is not due to the presence of isoenzymes displaying different affinities for substrates and inhibitors.

Several years ago we showed, by using acetylcholine (ACh) as a substrate, that the type of inhibition of serum cholinesterase (serum ChE; acylcholine acyl hydrolase, EC 3.1.1.8) by D-tubocurarine (TC) depends on the concentration of the inhibitor [1]. Recently we showed with the aid of the Dixon and the Cornish-Bowden [2] plots that in the linear range the inhibition is of the mixed type [3]. Non-linear inhibition suggested by the curved Lineweaver–Burk plot might indicate positive cooperativity. However the curve v–S is not sigmoidal. Another possible explanation is that isoenzymes, with different affinities for substrates and inhibitors are involved [4]. Since by its standard kinetic parameters ACh is not the most specific substrate for serum ChE, we decided to investigate the inhibition of this enzyme by TC using butyrylcholine (BuCh) and benzoylcholine (BzCh) as substrates. BuCh is the substrate for which serum ChE has the highest V_{max}, whereas BzCh is the substrate for which serum ChE has the smallest K_m thus being, according to Kalow [5], the most specific substrate for serum ChE.

MATERIALS AND METHODS

The two serum ChE preparations were: (1) horse serum (4 enzyme units per mg of protein), diluted 30-times in the assay sample; and (2) a filter cake obtained from the fifth stage of serum ChE purification according to Strelitz (220 enzyme units per mg of protein), concentration in the assay sample 10 μg per ml. BuCh iodide was used in concentrations from 0·5 mM to 20 mM, BzCh choloride from 1 mM to 10 mM, and TC chloride in concentrations from 0·005 mM to 1·0 mM. The substrates and inhibitor were obtained from Koch–Light.

Activity was measured with a Radiometer automatic titrator (Type TTT1c titrator, ABU1b automatic buret, TTA3 titration assembly, and SBR2c recorder). TC solution was added to the enzyme preparation in Ringer solution (0·154 M NaCl, 2·7 mM KCl, 2·3 mM $CaCl_2$, and 1 mM $MgCl_2$ with the addition of 0·1 M NaOH up to pH 7·4) without buffer. The reaction was started by addition of the substrate. In the experiments with the purified enzyme preparation, gelatine solution to the final concentration of 0·02% was added to Ringer solution. The volume of the reaction solution was 15 ml. The measurements were carried out at pH 7·4 and 25° in at least three parallel runs. The 10 mM solution of NaOH was prepared fresh evey day in a nitrogen atmosphere and bubbled through with nitrogen during measurements. A flow of nitrogen was also maintained over the surface of the reaction solution. The value of the non-enzymatic hydrolysis rate of the substrate was measured and subtracted from the value of the corresponding enzyme hydrolysis rate.

The dependence of the initial hydrolysis rate on TC concentration at different substrate concentrations was graphically analyzed according to Dixon and Cornish-Bowden [2].

RESULTS AND DISCUSSION

Inhibition by TC of BuCh hydrolysis catalyzed by serum ChE was of the same type in both native and purified enzyme preparations. The only difference was in the degree of inhibition: serum ChE in the diluted serum was about 20% more strongly inhibited than it was in the purified preparation with the same activity.

With BuCh as substrate the graphical analysis by plotting s/v vs i showed that the inhibition of serum ChE by TC is linearly mixed (Fig. 1). Recently, Cornish-Bowden [2] revealed that in the case of mixed inhibition the plot of s/v vs i and the plot of $1/v$ vs i complement one another. The straight lines at different substrate concentrations obtained by plotting s/v against i intersect at a point where the abscissa is $-K_i'$ and the ordinate $K_m(1 - K_i'/K_i)/V_{max}$,

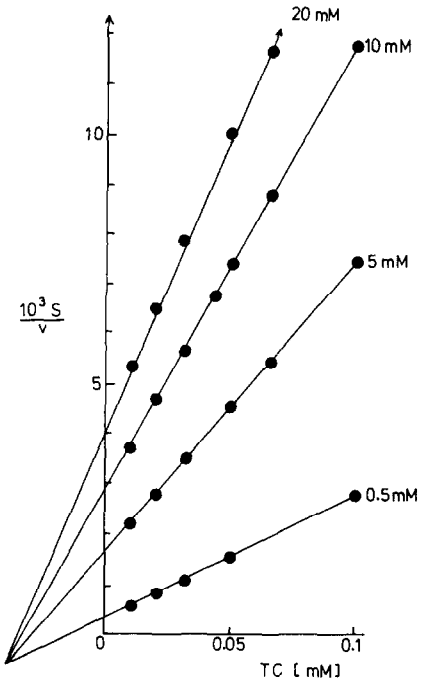

Fig. 1. Inhibition of horse serum cholinesterase by D-tubocurarine; substrate butyrylcholine; plot of s/v against i (Cornish-Bowden plot); pH 7·4; 25°.

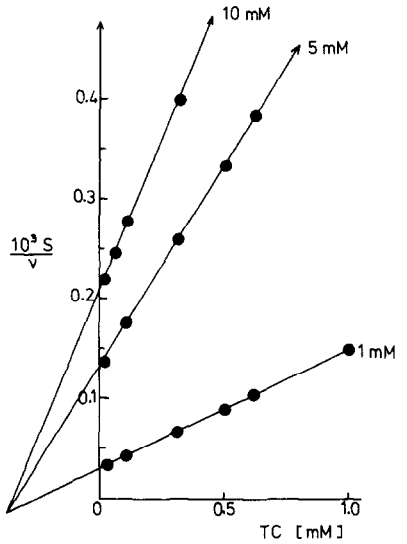

Fig. 2. Inhibition of horse serum cholinesterase by D-tubocurarine; substrate benzoylcholine; plot of s/v against i (Cornish-Bowden plot); pH 7·4; 25°.

whereas the intersection point of the lines of a Dixon plot for mixed inhibition has the coordinates: $i = -K_i$ and $1/v = (1 - K_i/K_i')/V_{max}$. Thus the two plots provide the measure of both K_i and K_i', which are in the case of mixed inhibition by TC of serum ChE-catalyzed hydrolysis of BuCh 8 μM and 40 μM, respectively.

With BzCh as substrate TC is a less potent inhibitor than with BuCh as substrate. Inhibition is likewise of the mixed type, K_i being 70 μM and K_i' 370 μM (Fig. 2). The ratio between K_i' and K_i is practically the same for both substrates: K_i' is about five times as large as K_i. The same ratio of inhibitor constants was obtained also with ACh as a substrate [3].

Thus the same type of enzyme inhibition by TC and a practically identical ratio between K_i' and K_i was obtained with both BuCh and BzCh. It might, therefore, be assumed that with different substrates the mechanism underlying the interaction of TC with the enzyme protein is very similar, if not identical. Mixed inhibition is obtained if the enzyme reacts with the substrate and the inhibitor so as to form a ternary complex (EIS). Recently, Bunting and Myers [6] showed that in the case of carboxypeptidase A partially competitive inhibition of esterase activity by the cyclohexylacetate ion might be explained as due to the formation of an EI_2 complex; analyzing the enzyme inhibition they obtained a linear Lineweaver–Burk plot a curved Dixon plot; replotting the reciprocal value of the initial velocity against the squared value of the inhibitor concentration, they obtained linear dependence. In the present case, however, the linear dependence obtained by both the method of Dixon and that of Cornish-Bowden precludes the possibility that mixed inhibition of serum ChE by TC is due to the formation of an EI_2 complex. We, therefore, assume that the inhibition of serum ChE by TC gives rise to the formation of an EI and an EIS complex. The existence of an EIS complex, when substrate and inhibitor do not react with each other, always points to an allosteric binding of the inhibitor to the enzyme protein. We hold that with substrates like BuCh and BzCh mixed inhibition of serum ChE by TC used in a wide range of concentrations can be explained in terms of an allosteric interaction of the enzyme protein with TC and not as due to the presence of isoenzymes with different affinities for TC. Thus the reaction between ChE and TC seems to resemble the reaction between AChE and TC for which Changeux [7] showed that it can be allosteric.

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REFERENCES

1. L. Kamarić, *Iugoslav. Physiol. Pharmacol. Acta* **6**, 107 (1970).
2. A. Cornish-Bowden, *Biochem. J.* **137**, 143 (1974).
3. L. Kamarić, *Iugoslav. Physiol. Pharmacol. Acta*, in press.
4. Y. C. Chiu, R. K. Tripathi and R. D. O'Brien, *Biochem. biophys. Res. Commun.* **46**, 35 (1972).
5. W. Kalow, *Can. J. Physiol. Pharmac.* **42**, 161 (1964).
6. J. W. Bunting and Ch. D. Myers, *Biochim. biophys. Acta* **341**, 222 (1974).
7. J.-P. Changeux, *Molec. Pharmac.* **2**, 369 (1966).