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INHIBITORS OF AROMATIC ESTERASE OF HUMAN SERUM

A. V. MARTON and W. KALOW

Department of Pharmacology, University of Toronto

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Abstract—Thirty-six drugs in a concentration of 10^{-4} were tested for influences on aromatic esterase activity. About one-third of them caused some inhibition. The three strongest inhibitors, in increasing order, were atropine, naphazoline, and chlorpromazine. The inhibition constant, K_i, of chlorpromazine was 3×10^{-5}. Twelve further phenothiazine derivatives showed an inhibitory potency of a similar magnitude, i.e., the inhibitory effect was nearly independent of molecular substitutions. Calcium did not influence the inhibition. Most inhibitors of aromatic esterase are known to be capable of influencing to various degrees other enzymes.

INTRODUCTION

THE name aromatic esterase was introduced by Mounter and Whittaker[1] because this enzyme hydrolysed esters of phenol and substituted phenols; this means that the enzyme is capable of combining with these agents. Since many drugs contain a phenol moiety we wondered whether these drugs also act as competitive inhibitors of aromatic esterase. Hence, the drugs first checked were related to phenol, but in the course of this investigation we came to include aromatic drugs of divergent structures.

A-esterase is a synonym for aromatic esterase.[2] It occurs in the albumin fraction of human serum.[3, 4] Resistance to inhibition by eserine and organophosphorus compounds is characteristic of this esterase.[1,2] Aldridge[2] found that the A-esterase not only is insensitive to organophosphorus compounds, but also it can hydrolyse at least one of them, diethyl *p*-nitrophenyl phosphate. Augustinsson and Olsson[5] selected eight compounds frequently employed as enzyme inhibitors and reported that the aromatic esterase from the plasma of some swine was resistant to all inhibitors tested. In a previous study[4] on separation of human A-esterase and pseudocholinesterase by electrophoresis, we found that barbital in a concentration of 8×10^{-3} M caused 50 per cent inhibition, while ethylenediaminetetra-acetic acid (EDTA; Versene) in a concentration of 5×10^{-5} completely blocked the activity of A-esterase. The activity blocked by EDTA could be restored with the aid of different bivalent metal ions.

EXPERIMENTAL

Assay of esterase activity

The Beckman Recording Spectrophotometer model DK2 was used for the determination of the activity of aromatic esterase. The activity was measured by recording the initial change of absorbance at 269 mμ which occurred during hydrolysis

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of phenyl acetate.[4, 6] The reaction was conducted in absorption cells with a light path of 1 cm at a temperature of 25·6 ± 0·3°C. The duration of a single measurement was 2 min.

Hydrogen-ion concentrations of the reaction mixtures were determined with a glass electrode in a Beckman pH-meter (model G) before and after each measurement; these values did not decrease more than 0·02 pH unit during the reaction. The solvent was always a mixture of equal parts of phosphate buffer (0·067 M, pH 7·4) and of tris-(hydroxymethyl)-amino-methane buffer (0·1 M, pH 7·4). This mixture of buffers was also used as an optical blank.

All measurements were carried out in quadruplicate. The average values of initial slopes were recorded.

Esterase

The sources of enzyme were samples of human serum which were free of haemolysed erythrocytes. The final dilution of crude serum was 1 : 400. Under these conditions the hydrolysis of phenyl acetate depends on the aromatic esterase activity but not on that of cholinesterase; the hydrolysis rate in crude serum was found to be proportional to that in the albumin fraction of the same sample of serum.[4]

Substrate

The substrate was a freshly prepared solution of phenyl acetate. Its initial concentration in the absorption cell was $3{\cdot}9 \times 10^{-4}$ M.

Esterase inhibitors

Forty-eight drugs were used as possible inhibitors. All the materials tested were compounds intended for medical use or commercially available products of equivalent purity. Stock solutions of all these compounds were prepared in a concentration of 4×10^{-4} M; the concentration in contact with the enzyme in the absorption cell was 1×10^{-4} M. The drugs were dissolved in buffer shortly before use and added simultaneously with the substrate to the serum.

RESULTS

Tables 1 and 2 show the results of the experiments with a standard concentration of 0·1 mM of various drugs. Fourteen of the forty-eight drugs examined did not have any influence on the rate of hydrolysis of phenyl acetate; eight of them caused a slight inhibition. The remaining twenty-six drugs inhibited the A-esterase in a range from 14 to 100 per cent.

All the barbiturates investigated, as well as thimerosal and methamphetamine, inhibited the hydrolysis of phenyl acetate between 10 and 20 per cent. Atropine, homatropine, neophenylephrine, dibucaine, papaverine and yohimbin caused a block of from 20 to 40 per cent. Fifty per cent or more inhibition was found with all the phenothiazine derivatives and with naphazoline, while EDTA completely blocked the enzymic hydrolysis.

The blocking effects of chlorpromazine and of naphazoline were investigated in greater detail. Experiments were carried out with these compounds in order to determine the dissociation constant of the enzyme–inhibitor complex, or in other words the

TABLE 1. EFFFCT OF DRUGS ON THE ACTIVITY OF AROMATIC ESTERASE

Compound	Synonyms	Percentage of original enzyme activity
Acetylsalicylic acid	Aspirin	>90
Amphetamine sulphate	1-Phenyl-2-aminopropane; benzedrine	100
Atropine sulphate	DL-Hyoscyamine	64
Azacyclonol HCl	α-(4-Piperidyl) benzhydrol; frenquel	85
Barbital Na	Barbitone; 5 : 5-diethylbarbituric acid	>90
Benzocaine HCl	Ethylamino-benzoate	100
Caffeine Na-benzoate	1 : 3 : 7-trimethylxanthine	100
Chlorpromazine HCl	Largactil; thorazine	31
Cocaine HCl	Methylbenzoyl ecgonine	100
Decamethonium bromide	Syncurine; C_{10}	>90
Dibucaine HCl	Cinchocaine; nupercaine HCl	75
EDTA	Ethylenediaminetetra-acetic acid; versene	0
Ephedrine sulphate		>90
Histamine phosphate		100
Homatropine HBr	Tropin mandelic ester	79
Iproniazid phosphate	*iso*Propyl isoniazid; marsilid phosphate	100
Mephenesin	Myanesin; lissephen; tolserol	100
Meprobamate	Miltown; equanil	>90
Methamphetamine HCl	Methedrine	86
Naphazoline HCl	α-Naphthyl-2-methylimidazoline; privine	49
Naphthylethylenediamine		100
Niamide	*N-iso*Nicotinoyl-N^1-(-*N*-benzylcarboxamido-ethyl)-hydrazine	100
DL-Norepinephrine HCl	Noradrenaline; arterenol; levophed	100
L-Norepinephrine bitartrate	L-Noradrenaline	100
Papaverine HCl		73
Pentylenetetrazol	Leptazol; cardiazol; metrazol	100
Phenylephrine HCl	Neosynephrine	75
Pilocarpine HCl		100
Procaine HCl	Novocaine	>90
Quinine HCl		>90
Secobarbital sodium	Seconal	83
Tetramethylammonium bromide	TMA	>90
Thimerosal	Merthiolate sodium	83
Thiopental sodium	Pentothal	82
Urethane	Ethyl urethane	100
Yohimbine HCl		69

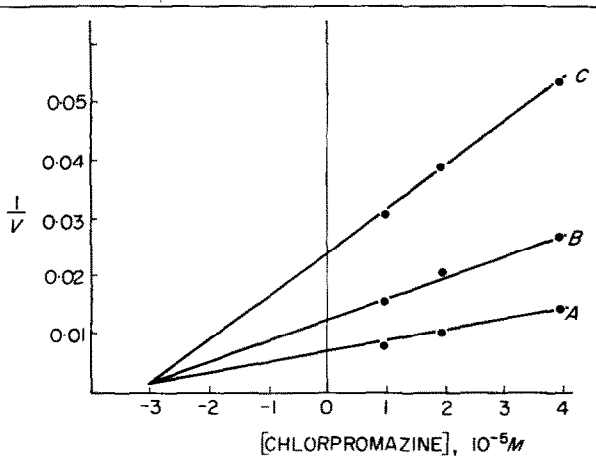
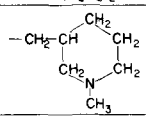
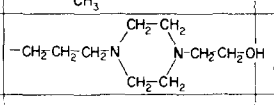
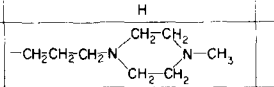
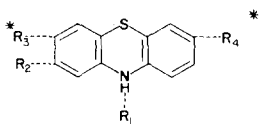


FIG. 1. Effects of chlorpromazine on aromatic esterase activity. The abscissae indicate molar concentrations of inhibitor, the ordinates give reciprocals of the hydrolysis rates of phenylacetate in arbitrary units. The concentration of phenylacetate was: $3{\cdot}7 \times 10^{-4}$ M for the data on line *A*; $1{\cdot}85 \times 10^{-4}$ M for the data on line *B*; $9{\cdot}25 \times 10^{-5}$ M for the data on line *C*.

inhibitor constant, K_i. The determination of K_i required measurements of initial velocities with a number of different concentrations of inhibitor and of substrate. The reciprocal of the velocity of the enzyme reaction ($1/V$) was plotted against the concentration of inhibitor (i), as described by Dixon.[7, 8] The results are shown in Fig. 1.

TABLE 2. EFFECT OF PHENOTHIAZINE DERIVATIVES ON THE ACTIVITY OF AROMATIC ESTERASE

Inhibitor	Synonym	Substituents of the Phenothiazine Nucleus*				Percent of original Enzyme activity
		R_1	R_2	R_3	R_4	
Chlorpromazine	Largactil	$-CH_2-CH_2-CH_2-N(CH_3)_2$	Cl	H	H	31
Diethyzine HCl	Diparcol	$-CH_2-CH_2-N(C_2H_5)_2$	H	H	H	34
Ethopropazine HCl	Parsitan	$-CH_2-CH(CH_3)-N(C_2H_5)_2$	H	H	H	43
Mepazine	Pacatal		H	H	H	29
Methylene blue**	Methylthionine	H	H	$-N(CH_3)_2$	$-N(CH_3)_2$	31
Levomepromazine	Nozinan	$-CH_2-CH(CH_3)-CH_2-N(CH_3)_2$	$-OCH_3$	H	H	25
Perphenazine	Trilafon		Cl	H	H	48
Phenothiazine HCl		H	H	H	H	39
Proclorperazine	Stemetil		H	Cl	H	24
Promethazine	Phenergan	$-CH_2-CH(CH_3)-N(CH_3)_2$	H	H	H	32
Tetrameprozine	Lispamol	$-CH_2-CH(N(CH_3)_2)-CH_2-N(CH_3)_2$	H	H	H	27
Triflupromazine	Vesprin	$-CH_2-CH_2-CH_2-N(CH_3)_2$	$-C(F)_3$	H	H	43
Trimeprazine	Panectyl Temaril	$-CH_2-CH(CH_3)-CH_2-N(CH_3)_2$	H	H	H	33



*The formula given here corresponds to the Leuco form of Methylene blue, though it was used in its oxidized form.

The K_i values obtained for chlorpromazine and naphazoline were $3{\cdot}15 \times 10^{-5}$ M and $6{\cdot}3 \times 10^{-5}$ M, respectively. Using the method of Hunter and Downs[9] for the evaluation of the experimental data the K_i values were: 4×10^{-5} M for chlorpromazine and $6{\cdot}9 \times 10^{-5}$ for naphazoline.

Five bivalent cations (Ca^{2+}, Zn^{2+}, Mg^{2+}, Mn^{2+}, Cu^{2+}) at a concentration of $2{\cdot}5 \times 10^{-4}$ M were capable of restoring the A-esterase activity which had been blocked completely by 10^{-4} M EDTA.[4] When 50 per cent of the original activity of A-esterase had been blocked by phenothiazine derivatives, no restoration could be achieved with the aid of $2{\cdot}5 \times 10^{-4}$ M Ca^{2+}.

DISCUSSION

In their studies on A-esterase of swine plasma, Augustinsson and Olsson[5] used the following inhibitors: physostigmine salicylate, tetra*iso*propylpyrophosphoramide, bis-mono*iso*propylaminofluorophosphine oxide (mipafox), 10-(1-diethylamino-propionyl)-phenothiazine hydrochloride (Astra 1397), 1:5-bis-(4-trimethylammonium phenyl)-pentane-3-one-di-iodide (62 C 47), bis-(piperidino methylcoumaranyl-5)-ketone (3318 CT), sodium arsanilate (Atoxyl) and quinine sulphate. They found that the enzyme was inhibited by none of these compounds. We have not tested Astra 1397, but judging from our results with other phenothiazine derivatives, it would probably have caused inhibition under our experimental conditions. We also found quinine to have some blocking effect. At present, one cannot decide whether these deviations from the results of Augustinsson and Olsson are due to a difference of species or methods. While we have not reported on physostigmine or organophosphorus inhibitors in this study, we have previously tested these inhibitors[4] with the same result as Augustinsson and Olsson.

In the experiments reported here, thirteen phenothiazines were investigated with different substituents on either the 10- or the 2-position or both, of halogenated and halogen-free derivatives. All these compounds showed a blocking effect of the same magnitude on aromatic esterase of human serum. Since the inhibitory effect of these compounds does not seem to be dependent on the substituent of the molecule, it is probably a property of the phenothiazine nucleus itself.

The most potent inhibitor of the tested drugs was EDTA, which has a fairly simple aliphatic structure. All other drugs which had a significant inhibitory effect on A-esterase of human serum were compounds of a more complicated chemical structure containing some kind of ring system in the molecule. EDTA acts as a chelating agent so that its inhibitory effects can be overcome by an excess of Ca^{2+} and some other cations; the inhibitory effect of the phenothiazine is refractory to calcium so that these agents act by a different mechanism.

Using graphical methods[7, 8] for the determination of the inhibitor constant, K_i, by plotting $1/V$ against i, a straight line is obtained; v represents the reaction velocity in the presence of a concentration, i, of the inhibitor at a given concentration of substrate. With another substrate concentration, a second straight line is obtained which intersects the first line. At this point of intersection one can obtain the value of K_i. This value lies above the base-line in case of competitive inhibition, and lies on the base-line in case of a non-competitive inhibitor. In the present work K_i values of chlorpromazine and of naphazoline were determined using this method. One cannot judge with certainty whether the inhibition is competitive or non-competitive: the intersection of the straight lines is close to the base-line, but the poor water solubility of phenyl acetate made it impossible to use the substrate in a concentration high enough to saturate the enzyme and to approach the maximum velocity (V_{max}). For the same reason it was not possible to make a reliable estimate of the Michaelis constant for phenyl acetate and aromatic esterase, either directly or through the K_i determinations.

No physiological function of A-esterase is known, and, with the exception of EDTA, no drug stands out as a particularly potent inhibitor of aromatic esterase. Relatively strong inhibiting drugs were atropine, naphazoline, and chlorpromazine; it is difficult to think of a common effect of atropine and naphazoline which could be

attributed to aromatic esterase inhibition. One may note that atropine,[10, 11] naphazoline,[12] and chlorpromazine[11, 13] are all capable of interacting with other enzymes. All these drugs can block both true and pseudocholinesterase; of the latter enzyme, both the usual form and the atypical variety[14] can be inhibited. In some rabbit sera, atropine is hydrolysed by atropine esterase.[15] Naphazoline has been shown to inhibit both mono- and di-aminoxidase.[12] Hence, there are several enzyme proteins known with which these drugs can combine *in vitro*, and most likely there are numerous further proteins. One feels compelled to assume that each drug *in vivo* occupies different proteins; the relative affinities characteristic for a drug and any given protein may then be said to determine its *receptor spectrum*.

After the manuscript of the present paper was completed, we noted a recent article by Erdös *et al.*[16] in which trivalent rare earth cations were described as the strongest inhibitors of aromatic esterase to-date.

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