Biochemical Pharmacology, Vol. 23, pp. 175–188. Pergamon Press, 1974. Printed in Great Britain.

SELECTIVE, NEAR-TOTAL, IRREVERSIBLE INACTIVATION OF PERIPHERAL PSEUDOCHOLINESTERASE AND ACETYLCHOLINESTERASE IN CATS *IN VIVO*

GEORGE B. KOELLE, RICHARD DAVIS, EMANUEL J. DILIBERTO, JR. and WINIFRED A. KOELLE

Department of Pharmacology, Medical School, University of Pennsylvania, Philadelphia, Pa. 19174, U.S.A.

(*Received* 10 *April* 1973; *accepted* 29 *June* 1973)

Abstract—Tetramonoisopropyl pyrophosphortetramide (Iso-OMPA) was confirmed to be a highly selective inactivator of cat pseudocholinesterase (BuChE) over wide ranges of concentration and temperature *in vitro*; intravenous doses of 3·0 or 6·0 μmoles (approximately 1·0 or 2·0 mg)/kg produced nearly total inactivation of peripheral BuChE, with no detectable inactivation of acetylcholinesterase (AChE). Selective inactivation of AChE *in vivo* was obtained as follows: after a dose of mephentermine, 2·0 to 3·0 mg/kg, i.v., to sustain blood pressure, cats received an intravenous dose of 10-(α-diethylaminopropionyl) phenothiazine HCl (Astra 1397) up to 200 μmoles (73 mg)/kg, which produces selective reversible inhibition of BuChE; they were then given 1·0 μmole (0·332 mg) 2-diethyoxyphosphinylthioethyldimethylamine acid oxalate (217 AO)/kg, which is sufficient when given alone to produce essentially total, irreversible inactivation of AChE and BuChE. Under these conditions, approximately one-third of the autonomic ganglionic BuChE, but none of the AChE, was protected and restored to activity. Quantitative results, obtained by a spectrophotometric method, were confirmed histochemically.

IN THE COURSE of developing the bis-(thioacetoxy) aurate (I) ($Au[TA]_2$) histochemical method[1,2] for the electron microscopic localization of acetylcholinesterase (AChE, EC 3.1.1.7) and pseudocholinesterase (BuChE, ChE, EC 3.1.1.8) in cat tissues, it became necessary to produce near-total, irreversible inactivation of each enzyme, with minimal inactivation of the other, in anesthetized cats prior to sacrifice. Of several organophosphorus anticholinesterase (anti-ChE) compounds that might be used for BuChE inactivation, tetramonoisopropyl pyrophosphortetramide (Iso-OMPA)[3,4] was found to be highly satisfactory. However, there appears to be no agent of this class that both penetrates cellular membranes readily and inactivates irreversibly AChE with sufficient selectivity. This end has been accomplished by the administration first of an appropriate intravenous dose of 10-(α-diethylaminopropionyl) phenothiazine HCl (Astra 1397), a highly selective reversible inhibitor of BuChE,[5] followed by a dose of 2-diethoxyphosphinylthioethyldimethylamine acid oxalate (217 AO) sufficient, when given alone, to produce essentially complete, irreversible inactivation of both AChE and BuChE;[6] in this sequence, a considerable proportion of the BuChE, but none of the AChE, is protected and subsequently restored to activity. These procedures should be applicable also to a variety of physiological and pharmacological

investigations of the functions of AChE and BuChE, and hence are reported and substantiated in full.

METHODS

Determination of AChE and BuChE activities

The method of Ellman *et al.*[7] was employed, using a Beckman DU quartz spectrophotometer, and all determinations were performed at 30° in duplicate. The reaction mixtures for AChE determinations consisted of final concentrations of $3{\cdot}3 \times 10^{-5}$ M Astra 1397, water to give a final volume of 3·0 ml, 0·067 M phosphate buffer, final pH 7·9, homogenate of tissue or dilution of plasma, $3{\cdot}3 \times 10^{-4}$ M 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), and 0·003 M acetylthiocholine iodide (AThCh), added to the cuvettes in that sequence. For BuChE determinations, $3{\cdot}3 \times 10^{-6}$ M 1,5-bis-(4-allyl dimethylammonium phenyl) pentan-3-one dibromide (B.W. 284)[4,8] was substituted for Astra 1397, and 0·01 M butyrylthiocholine iodide (BuThCh) for AThCh. Readings were taken at 412 nm at 1-min intervals for 12 min, and activities were calculated by subtracting the 2-min from the 12-min readings; values were corrected for spontaneous hydrolysis.

Potencies of inhibitors in vitro

Determinations were conducted with homogenates of cat jejunum-ileum and of autonomic ganglia for ACh and BuChE, and of brainstem for AChE.

The jejunum-ileum was removed under anesthesia with sodium pentobarbital (30 mg/kg, intraperitoneally); it was stripped of adjacent tissue, slit open, washed rapidly with running tap water, blotted, weighed, and scissor-minced in a beaker held in an ice-water bath; after the addition of four parts of cold water, it was homogenized with a Polytron PT 10 (Brinkmann Instruments, Inc.) at 11,000 rev/min, for two 2-min periods in an ice-water bath; aliquots were then frozen. At the time of use, an aliquot was thawed in a water bath at room temperature, and an equal volume of cold water was added; it was then rehomogenized for 2 min as above, and strained through four layers of gauze in the refrigerator.

Stellate and superior cervical ganglia were weighed, pooled, and frozen immediately after removal, then thawed and homogenized similarly with 250–500 parts of water for four 1-min periods. The homogenates were frozen until use, at which time they were thawed and resuspended with a glass hand-homogenizer.

The brain was removed immediately after sacrifice of the anesthetized cat by exsanguination; the brainstem plus the caudate nuclei were weighed, homogenized with nine parts of water for 2 min as above, and aliquots were frozen. Aliquots were thawed and resuspended with a glass hand-homogenizer immediately prior to use.

Since the irreversible inactivation of AChE or BuChE by the organophosphorus compounds, Iso-OMPA and 217 AO, involves relatively slow, bimolecular reactions,[9] the time and temperature of the reactions must be controlled to obtain meaningful, reproducible data. This was done as follows: to each of a series of flasks, held in an ice-water bath, were added 2·0 ml homogenate, 2·0 ml of 0·1 M phosphate buffer, pH 8·0, and water, to give a final volume of 10·0 ml after the addition of inhibitor. As soon as a freshly prepared aqueous solution of Iso-OMPA or 217 AO was added, each vessel was transferred to a 37° or 5° water bath and swirled rapidly for temperature

equilibration. At the end of the incubation period (15 min at 37°, or 60 min at 5°), 1·0 ml of 0·1 M acetylcholine chloride (ACh) was added (final concentration 0·0091 M) to halt the reaction, and the vessels were kept in an ice-water bath until assay shortly thereafter. In order to simulate as closely as possible conditions present *in vivo*, or with tissue sections *in vitro* when Iso-OMPA was used for the selective inhibition of BuChE, an additional series was run using the stellate-superior cervical ganglion homogenate (2·0 ml) as the enzyme source; to this was added only Krebs–Ringer solution[10] and Iso-OMPA (2×10^{-3} M in Krebs–Ringer solution) to a total volume of 10·0 ml prior to incubation; the procedure was otherwise identical with that described above.

For determinations of the potencies of the reversible inhibitors, B.W. 284 and Astra 1397, the compounds were added directly to the cuvettes in the same sequence as for the routine enzyme assays, as described above. Since BuThCh, like butyrylcholine, is hydrolyzed at a practically insignificant rate by AChE,[11,12] inhibition of its hydrolysis in the presence of homogenates containing both enzymes could be equated approximately with BuChE inhibition. For the measurement of AChE inhibition, homogenates of jejunum-ileum and brainstem were exposed to 10^{-4} M Iso-OMPA at 5° for 60 min and the reaction was halted with 0·0091 M ACh, as above; the mixtures were transferred to cellulose dialysis tubes and dialyzed against cold, running tap-water overnight. The contents were then hand-homogenized and used as a source of AChE free of BuChE, as demonstrated by their failure to hydrolyze BuThCh at a significant velocity.

Selective protection of BuChE by Astra 1397 *against* 217 *AO* in vitro

The procedure followed here was similar to that employed for determining the anti-ChE potencies of Iso-OMPA and 217 AO. To flasks in an ice-water bath were added successively aliquots of jejunum-ileum homogenate, buffer, and water; graded concentrations of Astra 1397 were then added and the vessels were transferred to a 37° water bath; exactly 5 min later, 217 AO was added to produce a final concentration of 10^{-6} M and the mixtures were incubated for an additional 15 min at 37°. After the addition of ACh (to 0·0091 M) and cooling, the contents of the vessels were transferred quantitatively to centrifuge tubes and centrifuged at approximately 3000 *g* for 60–75 min in a Sorvall angle centrifuge in the cold room at 5°. The supernatant portions were decanted, the sediments resuspended in 10·0 ml of 0·02 M phosphate buffer, pH 8·0, and centrifugation was repeated. After decanting the supernatant portion, the sediment of each tube was resuspended in 5·0 ml H_2O, hand-homogenized, and kept in an ice-water bath until assay. Activities of AChE and BuChE were expressed as percentages of the control sample to which no Astra 1397 or 217 AO had been added.

Attempts to remove the inhibitors from the reaction mixtures by dialysis overnight were unsatisfactory, because most of the AChE and BuChE activities of the controls were lost by autolysis; the BuChE activity of the test mixture which had contained the highest concentration of Astra 1397 (10^{-4} M) prior to the addition of 217 AO was over four times that of the control, probably because 217 AO can inactivate the autolytic enzymes. However, this was accomplished by using as the enzyme source cat plasma, which contains significant concentrations of both BuChE and AChE, and apparently considerably less proteolytic activity. Following the same initial steps

through the addition of ACh, as above, 5·0-ml aliquots of the reaction mixtures were transferred to cellulose dialysis tubes, which were placed directly into a cold, 500-ml bath containing 0·01 M ACh and 0·01 M phosphate buffer, pH 8·0. Dialysis was continued against ice-cold running tap water overnight; then the contents were transferred quantitatively to graduate cylinders, brought with rinsings to 6·5 ml, hand-homogenized and assayed.

Selective inactivation of BuChE and AChE in vivo

General procedure and controls. Activities of AChE and BuChE were determined quantitatively with the superior cervical, stellate and ciliary ganglia, the ileum, caudate nucleus and plasma; they were monitored histochemically by a modification of the copper thiocholine (CuThCh) method[13] in the same tissues, with the exception that the carotid body was included in place of plasma.

Control values were obtained from three cats, two prior to and one after the experimental series. After anesthesia with sodium pentobarbital (30 mg/kg, intraperitoneally), the animals were exsanguinated through a femoral arterial cannula; the first portion of blood was heparinized, centrifuged in the cold room, and the plasma removed and frozen. One set of ganglia, a 1-cm segment of ileum immediately craniad to the ileo–cecal junction, and a portion of caudate nucleus were blotted, weighed on a Mettler H2OT balance, and frozen as rapidly as possible. The other set of ganglia, the adjacent portion of ileum, a portion of caudate nucleus and the carotid bodies were placed immediately in cold formaldehyde (4%)–Krebs–Ringer solution;[10] after 3 hr, they were transferred to cold Krebs–Ringer solution, and kept for sectioning and staining the following day. The frozen samples were transferred to cold homogenizing tubes, and cold water was added in the following proportions: superior cervical and stellate ganglia, 500:1; ciliary ganglion, 3000:1; ileum, 39:1; caudate nucleus, 200:1. The sample of ileum was first scissor-minced, then transferred quantitatively with rinsings; after homogenization, it was strained through four layers of gauze prior to freezing. Homogenization was conducted with an ice-water bath surrounding the tubes, using the Polytron PT 10, 11,000 rev/min, for the following exact time periods: ganglia, 4 × 1 min; ileum, 3 × 2 min; caudate nucleus, 2 min. Homogenates were frozen immediately; the following day they were thawed, resuspended with a glass hand-homogenizer, and assayed by the method described above. The thawed plasma was strained through gauze and diluted (1 + 9 parts water) prior to assay. Control values are expressed as nmoles of AThCh or BuThCh hydrolyzed/mg wet weight of tissue for 10 min. Experimental values (see below) are expressed as percentages of the mean control values.

For histochemical examination, frozen sections of each tissue were cut at 10 μm with a cryostat microtome and distributed sequentially on four slides (eight for the stellate ganglion). One-half the slides were immersed in 3×10^{-4} M or 10^{-3} M Iso-OMPA in Krebs–Ringer solution at 5° for 60 min for the selective inactivation of BuChE; they were rinsed for 10 min in Krebs–Ringer solution at 5°, twice in water at 5° for 5 min each, then dried in air. The major differences between the CuThCh staining method used here and the standard method[13] were the pH of the pre-incubation, incubation, and rinse solutions (5·50 to 5·55), and the use of an acidified (0·2 ml glacial acetic acid/15·0 ml) solution of 2·5% $Na_2S \cdot 9H_2O$ in 6% Na_2SO_4 for converting the precipitate of CuThCh-sulfate to CuS; all steps were conducted at room temperature. After

30 min in pre-incubation solution, an untreated and Iso-OMPA treated slide of each tissue were immersed for 2 hr in AThCh-incubation solution (for the localization of AChE plus BuChE, and AChE only, respectively) and a similar pair was incubated in BuThCh-incubation solution (for BuChE and the control respectively). The additional set of stellate ganglion slides was incubated for 1 hr. Slides were then carried through the rinse solutions, developed in Na_2S for 2 min, gold-toned, dehydrated and mounted.

Iso-OMPA. After preliminary trials, the selective inactivation of BuChE was found to be produced by the intravenous injection of 3·0 μmoles (1·03 mg) or 6·0 μmoles (2·06 mg) Iso-OMPA/kg. Cats were prepared and the tissues treated as in the control series. They were sacrificed 15 min after the injection of 3·0 or 6·0 ml/kg of a 10^{-3} M solution of Iso-OMPA in 0·9% NaCl over a period of 30–60 sec. Tissue samples were taken and treated as in the control series.

Astra 1397 *plus* 217 *AO.* The procedure employed in the final experiments, described below, was developed in the course of the series. The necessity of recording the EKG and blood pressure and administering a vasopressor agent, mephentermine, was entailed by the precipitate fall in blood pressure that followed the intravenous injection of doses of Astra 1397 in excess of 50 μmoles (18·2 mg)/kg.

After anesthesia with sodium pentobarbital, the trachea was cannulated and artificial respiration applied with a Palmer pump. A catheter was introduced into the femoral artery for recording blood pressure by means of transducer coupled with a Physiograph, type DMP-4A (E & M Instrument Co., Houston); the electrocardiogram was recorded from standard limb lead II. An infusion of 5% glucose was given via the femoral vein, with a three-way stopcock interposed for the injection of all subsequent drugs. Approximately 10 min after the injection of atropine, 1 mg/kg, an injection of mephentermine sulfate, 2·0 to 3·0 mg/kg, was given, which produced an immediate, sustained rise in blood pressure; at the peak, approximately 1 min after injection, the dose of Astra 1397 (0·01 M in 0·9% NaCl) was injected over 3 min. One and one-half min after the injection of Astra 1397, 217 AO was injected over 0·5 min, in a dose of 0·5 μmole (166 μg) or 1·0 μmole (332 μg)/kg.

The animal was maintained for 15–64 min; after the collection of an arterial blood sample, artificial respiration was terminated and the same tissues were taken and treated as above. In some of the earlier experiments, in which high doses of Astra 1397 were given and mephentermine was not employed, death occurred sooner.

RESULTS

Inactivation or inhibition of AChE and BuChE in vitro

Iso-OMPA produced nearly complete inactivation of BuChE, with no detectable inactivation of AChE, in the jejunum-ileum homogenate after incubation at 37° for 15 min over the concentration range of 3×10^{-6} to 3×10^{-5} M; at 10^{-4} M and higher, progressive inactivation of AChE occurred (Table 1). Selectivity was even more marked when incubation was conducted at 5° for 60 min; here BuChE was essentially totally inactivated, and AChE unaffected over the range of 10^{-5}–10^{-3} M. Results were identical over the concentration range employed with the homogenate of stellate-superior cervical ganglia and Krebs–Ringer solution (Table 2). These conditions of temperature and time were selected to simulate, respectively, those obtained

in vivo when Iso-OMPA was injected intravenously and tissues were taken 15 min later, and *in vitro* when tissue sections were treated for the selective inactivation of BuChE.

TABLE 1. SELECTIVE INACTIVATION OF BuChE OF CAT JEJUNUM-ILEUM BY TETRAMONOISOPROPYL PYROPHOSPHORTETRAMIDE (ISO-OMPA)*

Iso-OMPA (M)	Control activity (%)			
	37°, 15 min		5°, 60 min	
	AChE	BuChE	AChE	BuChE
10^{-8}	99	94		
10^{-7}	99	70		
3×10^{-7}	96	36		
10^{-6}	103	8·3	104	46
3×10^{-6}	105	3·2	109	12
10^{-5}	101	2·3	103	1·7
3×10^{-5}	100	1·4	107	1·0
10^{-4}	91	1·1	100	0·1
3×10^{-4}	82	1·2	105	0
6×10^{-4}	78	0	92	1·6
10^{-3}	64	0·5	94	1·1

* See text for details.

TABLE 2. SELECTIVE INACTIVATION OF BuChE OF CAT SUPERIOR CERVICAL-STELLATE GANGLIA BY TETRAMONOISOPROPYL PYROPHOSPHORTETRAMIDE (ISO-OMPA)*

Iso-OMPA (M)	Control activity (%)			
	37°, 15 min		5°, 60 min	
	AChE	BuChE	AChE	BuChE
10^{-4}	94	0		
3×10^{-4}	82	0	104	0
6×10^{-4}	71	0	106	0
10^{-3}	57	0	98	0

* See text for details.

217 AO showed definite selectivity against BuChE over the concentration range tested; however, after 15 min of incubation at 37° with 10^{-6} M, both BuChE and AChE were essentially totally inactivated (Table 3).

B.W. 284 produced complete inhibition of AChE, with no detectable inhibition of BuChE at 10^{-6} M; as the concentration was increased beyond this, there was progressive inhibition of the latter enzyme (Table 4).

Astra 1397, in contrast, brought about nearly total inhibition of BuChE at 10^{-4} M, at which concentration there was only 10 per cent inhibition of AChE; at 3×10^{-5} M, over 90 per cent of the BuChE was inhibited and AChE was unaffected (Table 5).

TABLE 3. INACTIVATION OF CAT AChE AND BuChE BY 2-DIETHOXY-PHOSPHINYLTHIOETHYLDIMETHYLAMINE ACID OXALATE (217 AO)

217 AO (M) (37°, 15 min)	Control activity (%)		
	AChE		BuChE
	Brain	Jejunum-ileum	Jejunum-ileum
3×10^{-8}	75	84	9·3
10^{-7}	33	40	4·9
3×10^{-7}	13	6·8	6·2
10^{-6}	5·3	0	0

TABLE 4. SELECTIVE INHIBITION OF CAT AChE BY 1,5-BIS-(4-ALLYL-DIMETHYLAMMONIUM PHENYL) PENTAN-3-ONE DIBROMIDE (B.W. 284)

B.W. 284 (M)	Control activity (%)		
	AChE		BuChE
	Brain	Jejunum-ileum	Jejunum-ileum
10^{-6}	0	0	99
3×10^{-6}	0	0	94
10^{-5}	0	0	88
3×10^{-5}	0	0	74
10^{-4}	0		37

TABLE 5. SELECTIVE INHIBITION OF CAT BuChE BY 10-(α-DIETHYLAMINO-PROPIONYL) PHENOTHIAZINE HYDROCHLORIDE (ASTRA 1397)

Astra 1397 (M)	Control activity (%)		
	AChE		BuChE
	Brain	Jejunum-ileum	Jejunum-ileum
10^{-6}	98		53
3×10^{-6}	101	103	35
10^{-5}	99	99	14
3×10^{-5}	102	100	8
10^{-4}	90	89	3

Protection of BuChE by Astra 1397 *against* 217 *AO* in vitro

In the experiments where the homogenate of jejunum-ileum was used and the inhibitors were removed by centrifugation, there were recoveries of 26 per cent of BuChE and 32 per cent of AChE in the control samples (no Astra 1397 or 217 AO) in terms of the original homogenate. The corresponding recovery values in the plasma experiments where the samples were dialyzed overnight were 48 per cent for BuChE and 11 per cent for AChE. In the former series, the losses probably reflect the portions

of the enzymes that were in solution or associated with particles too small to be recovered under the conditions of centrifugation employed; in the latter, they resulted undoubtedly from autolysis, which was nevertheless considerably less than with jejunum-ileum homogenates under the same conditions. As in the previous experiments, exposure to 10^{-6} M 217 AO alone at 37° for 15 min resulted in essentially complete inactivation of BuChE and AChE (Table 6). With the prior addition of 10^{-4} M Astra 1397, a mean (from two experiments) of 71 per cent of BuChE was protected with the jejunum-ileum homogenate, and 57 per cent with the plasma, in terms of the controls. At 10^{-5} M Astra 1397, approximately 10 per cent of the BuChE was protected in both types of experiments; there was no detectable protection of BuChE at lower concentrations. No protection of AChE was detected at any concentration of Astra 1397 in either type of experiment. The absence of protection of AChE was confirmed in a dialysis experiment over the same range of concentrations of Astra 1397, in which the brainstem–caudate nuclei homogenate was used as the enzyme source; here, however, the recovery of BuChE in the control was too low to permit demonstration of its protection.

TABLE 6. PROTECTION OF BuChE AGAINST 10^{-6} M 217 AO BY ASTRA 1397 *in vitro**

Astra 1397 (M)	Recovery (%)			
	Jejunum-ileum (centrifugation)		Plasma (dialysis)	
	BuChE	AChE	BuChE	AChE
0	3	2	3	0
10^{-6}	4	1		
3×10^{-6}	4	2		
10^{-5}	10	1	11	0
3×10^{-5}	60	2	14	0
10^{-4}	71	0	57	0

* See text for details.

The percentage inhibition of BuChE (Table 5) and percentage protection against 217 AO (Table 6) were plotted against the negative logarithms of the molar concentrations of Astra 1397. From the curves so obtained, it was estimated that 25 to 100 times the dose producing 50 per cent inhibition was required for the same degree of protection in the centrifugation and dialysis experiments respectively.

Selective inactivation of BuChE and AChE in vivo

Quantitative measurements after Iso-OMPA. The autonomic ganglia removed from cats sacrificed approximately 15 min after the intravenous injection of 3·0 μmoles Iso-OMPA/kg showed residual BuChE activity ranging from 0·2 to 3·4 per cent of control values, and slightly higher residual activity (2·1 to 5·6 per cent) for the ileum and plasma (Table 7). There was no detectable inactivation of AChE; values in excess of 100 per cent, particularly for the ileum, reflect the marked range of values noted in the three control cats. After 6·0 μmoles Iso-OMPA/kg, the mean value for

TABLE 7. SELECTIVE INACTIVATION OF BuChE BY ISO-OMPA IN CATS *in vivo**

	Controls (nmoles AThCh or BuThCh/mg/10 min)				Iso-OMPA (% controls)					
					3·0 μmoles/kg			6·0 μmoles/kg		
	1	2	3	Means	1	2	Means	1	2	Means
AChE										
Superior cervical ganglion	208	176	164	183	90	136	113	82	121	102
Stellate ganglion	167	101	126	131	106	139	123	83	132	108
Ciliary ganglion	149	155	241	182	67	102	85	55	132	94
Ileum	8·1	13·5	9·7	10·4	200	149	175	190	153	172
Caudate nucleus	607	677	446	573	124	107	116	96	104	100
Plasma	2·3	1·6	1·7	1·9	134	132	131	96	110	103
BuChE										
Superior cervical ganglion	576	199	233	336	2·2	0·8	1·5	0	1·6	0·8
Stellate ganglion	509	187	231	309	2·2	0·2	1·2	0	1·5	0·8
Ciliary ganglion	137	177	185	166	0·7	3·4	2·1	0	0	0
Ileum	15·1	21·4	19·1	18·5	2·1	5·6	3·9	4·2	3·9	4·1
Caudate nucleus	29·3	36·2	25·9	30·5	113	60	87	32	61	47
Plasma	12·6	9·7	8·3	10·2	4·2	2·9	3·6	1·5	2·1	1·9

* See text for details.

BuChE in each of the three ganglia of the two cats was less than 1 per cent, and the highest individual value was 1·6 per cent; AChE was still essentially unaffected. Thus, this represents nearly total selective inactivation of ganglionic BuChE. That the blood–brain barrier restricts the penetration of Iso-OMPA into the central nervous system is indicated by the relatively high mean residual values for BuChE in the caudate nucleus after the two doses, namely 87 and 47 per cent.

Quantitative measurement after Astra 1397 *plus* 217 *AO.* In the earlier series, where 217 AO was given in a dose of 0·5 μmole/kg, no protection of BuChE was detected after 30 μmoles Astra 1397/kg (Table 8). Some degree of protection was produced in all tissues, but not in plasma, after 50 μmoles/kg, and marked protection was obtained after 70 and 100 μmoles/kg. However, the dose of 217 AO employed failed to achieve consistently the desired degree of inactivation of AChE in all tissues. Accordingly, this was raised to 1·0 μmole/kg, which produced 97 per cent to essentially total inactivation of AChE in the ganglia, and nearly the same values for the ileum and caudate nucleus. With the doubling of the dose of 217 AO, it was necessary to increase the preceding dose of Astra 1397 by more than this increment in order to obtain equivalent protection of BuChE. Thus, no significant protection was noted after 50 μmoles/kg; protection was slight after 70 and 100, and consistent for all tissues only after 140 μmoles Astra 1397/kg. After 200 μmoles Astra 1397/kg, the mean value for protection of BuChE in the three ganglia was approximately 35 per cent in both cats, sacrificed 60 min and 15 min, respectively, after 217 AO; values for the ileum and caudate nucleus were somewhat lower. No protection of plasma BuChE was obtained in any animals of this entire series.

Histochemical confirmation. Findings with all six tissues studied were comparable with those demonstrated for the stellate ganglion in Fig. 1. When sections prepared from a control cat were incubated in the AThCh medium at room temperature for 2 hr, intense overstraining, indicated by heavy deposits of acicular crystals, was produced at sites of both AChE and BuChE activity (Fig. 1, panel 1). Prior incubation of the sections in 10^{-3} M Iso-OMPA at 5° for 60 min for selective inactivation of BuChE, followed by the foregoing procedure, resulted in staining only at sites of AChE activity, in the neuropil and to varying degrees of intensity in the perikarya of the ganglion cells (Fig. 1, panel 2). Incubation of untreated control sections in the BuThCh medium under the same conditions caused selective overstaining of BuChE activity in the areas between ganglion cells (Fig. 1, panel 3). When this treatment was preceded by incubation with Iso-OMPA, as above, the sections remained blank (Fig. 1, panel 4). These results are identical with those reported in numerous earlier studies involving various modifications of the same general procedure and a variety of selective inhibitors of AChE and BuChE.[12–14]

In the stellate ganglion from a cat that had received 6·0 μmoles Iso-OMPA/kg, i.v., 15 min prior to sacrifice, the pattern of staining after incubation with AThCh, without or with prior treatment with Iso-OMPA *in vitro* (Fig. 1, panels 5 and 6), was identical with the AChE pattern of the control cat (Fig. 1, panel 2). The section stained for BuChE was blank (Fig. 1, panel 7) as was the control (Fig. 1, panel 8).

Results in the tissues of a cat that had received Astra 1397 (200 μmoles/kg) followed by 217 AO (1·0 μmole/kg), i.v., 63 min prior to sacrifice were consistent with the presence of BuChE only, in reduced concentrations. Sections incubated with either AThCh (Fig. 1, panel 9) or BuThCh (Fig. 1, panel 11) were stained in a pattern

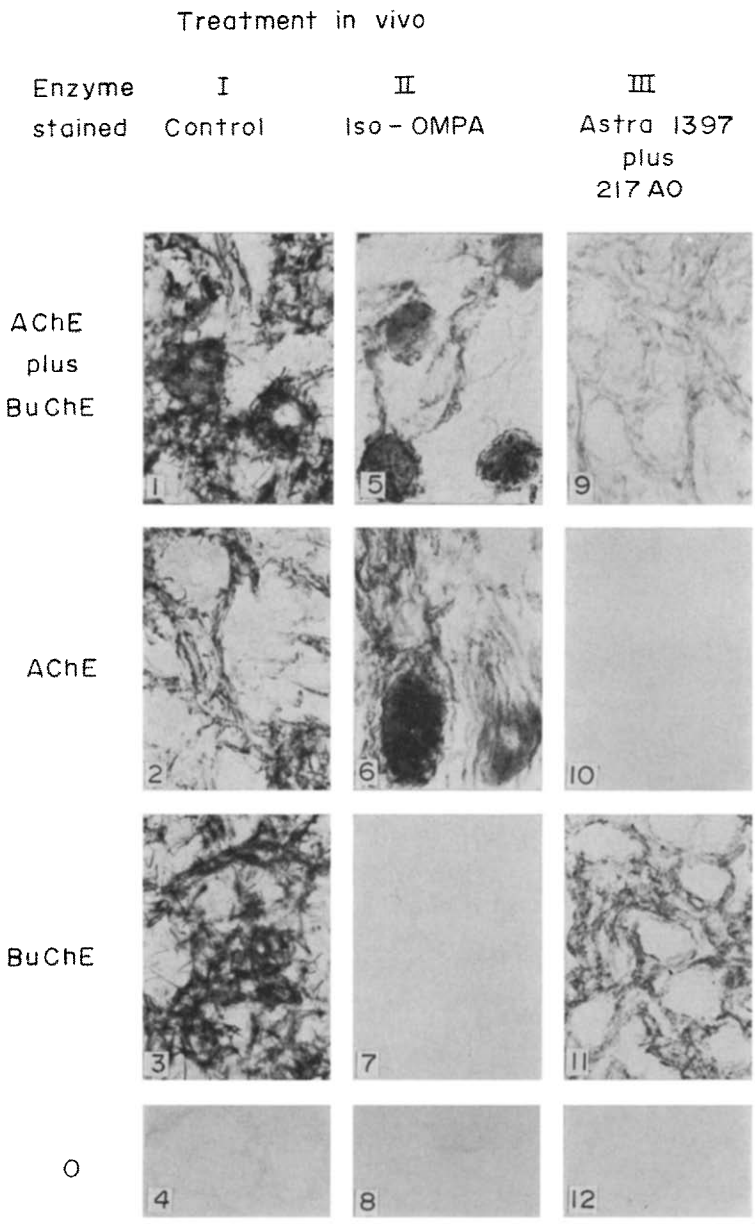

FIG. 1. Sections (10 μm) of cat stellate ganglia stained by CuThCh method for AChE plus BuChE (AThCh), AChE (Iso-OMPA → AThCh), BuChE (BuThCh) and 0-controls (Iso-OMPA → BuThCh), by incubation with substrate indicated for 2 hr at room temperature, with or without prior incubation with 10^{-3} M Iso-OMPA, 5°, 60 min, for selective inactivation of BuChE. Magnification (× 270). (I) Control cat (panels 1–4). (II) Iso-OMPA, 6·0 μmoles/kg, i.v., for selective inactivation of BuChE (panels 5–8). (III) Astra 1397, 200 μmoles/kg, i.v., plus 217 AO, 1·0 μmoles/kg, i.v., for selective inactivation of AChE (panels 9–12).

B.P.—*facing page* 184

TABLE 8. SELECTIVE INACTIVATION OF AChE BY ASTRA 1397 PLUS 217 AO IN CATS *in vivo**

	Controls (%)									
	0·5 μmole 217 AO/kg				1·0 μmole 217 AO/kg					
	μmoles Astra 1397/kg									
	30	50	70	100	50	70	100	140	200	200
AChE										
Superior cervical ganglion	7·4	2·5	3·1	0·4	2·8	0·3	0·2	0·4	1·2	1·6
Stellate ganglion	8·1	5·5	1·7	0·3	1·4	0	0	3·1	3·1	1·4
Ciliary ganglion		3·7	8·7	0	0	0	0	0	0	0·6
Ileum	0	6·1	4·8	0	0	0·9	0	3·9	3·9	3·0
Caudate nucleus	4·9	5·5	62·5	30·3	4·7	3·9	0	3·6	2·4	3·9
Plasma	5·1	6·1	2·4	2·4	7·3	12	3·6		8·1	10
BuChE										
Superior cervical ganglion	3·0	8·0	60	37	1·5	7·4	15	16	28	37
Stellate ganglion	3·7	8·4	61	72	2·9	11	18	23	31	33
Ciliary ganglion		24	122	57	2·7	11	12	43	48	37
Ileum	3·4	9·2	60	42	4·6	6·6	4·4	9·7	21	13
Caudate nucleus	8·0	20	145	83	13	15	8·6	22	23	27
Plasma	2·2	3·3	2·3	3·7	2·9	3·1	1·4		4·6	3·5
Time of sacrifice or death (min)	63	64	< 10	< 10	62	64	13	63	63	15

* See text for details.

identical with that of the BuChE-stained sections of the control cat (Fig. 1, panel 3); the intensity of staining with BuThCh was considerably greater than that with AThCh, reflecting the greater velocity of hydrolysis of the former substrate by BuChE. Sections treated with Iso-OMPA *in vitro* prior to incubation with either substrate were blank (Fig. 1, panels 10 and 12).

Acute pharmacological effects. In anesthetized cats that had received no other drugs, the intravenous injection of 3·0 or 6·0 μmoles (approximately 1 or 2 mg) Iso-OMPA/kg over 0·5 min produced no detectable effects on blood pressure or on the appearance of the EKG; likewise, no changes in respiration were noted, and there was no appearance of skeletal muscular fasciculation or twitching. The only suggestion of any effect was a possible increase in intestinal motility as observed externally through the abdominal wall, but this was not investigated further.

The major, and limiting, actions of Astra 1397 over the dosage range employed were on the cardiovascular system. After a dose of atropine, ranging from 10·0 mg/kg in the early experiments to 1·0 mg/kg, i.v., in the final series, an intravenous injection of 30·0 μmoles (10·9 mg) Astra 1397/kg or higher caused an immediate, precipitate fall in blood pressure. Spontaneous recovery followed 30 or 50 μmoles/kg, so that animals could be maintained and sacrificed an hr or more later. After a dose of 70 μmoles (25·4 mg)/kg or greater, the blood pressure continued to fall and death occurred within 10 min or less. However, fatal cardiovascular shock ordinarily brought about by doses of Astra 1397 up to 200 μmoles (72·6 mg)/kg could usually be prevented by the prior intravenous injection of 1·5 to 3·0 mg mephentermine (as the sulfate)/kg. At the peak of the hypertensive response to mephentermine (1–2 min after injection), the dose of Astra 1397 was injected over a period of 3 min; while the blood pressure still fell precipitately, it then gradually rose and became stabilized in most cases. No further changes were noted after the injection of 217 AO, 0·5 or 1·0 μmole (0·166 or 0·332 mg)/kg, approximately 1·5 min after the injection of Astra 1397. Although this dosage range of 217 AO usually produces generalized skeletal muscular fasciculation in anesthetized, atropinized cats, this was never noted in the Astra 1397-pretreated animals.

Accompanying the foregoing changes in blood pressure was a wide spectrum of changes in the EKG, ranging from minor slowing of the normal sinus rhythm to cessation of all electrical cardiac activity, save for occasional ectopic ventricular beats. Changes in the EKG attributable to Astra 1397 and/or 217 AO on cardiac function consisted mainly of slowing of the normal sinus rhythm, an increased P-R interval, and evidence of impaired conduction through the ventricular conduction system, as reflected by changes of QRS pattern and increased conduction time. In some cats, ectopic ventricular complexes were noted, varying from occasional to frequent. Periods of nodal rhythms alternating between high and low nodal also were observed; in one case, complete AV dissociation followed. The more serious changes were always associated with the precipitous drops in blood pressure discussed above.

The only additional notable pharmacological effect in this series of cats was a marked reduction in the output of urine. The bladder was catheterized and urine flow measured from the beginning of the experiments; in spite of the infusion on some occasions of several hundred ml of 5% glucose solution over the course of the hr following the drugs, no more than 20–30 ml urine was collected. In such instances,

the gastrointestinal tract was markedly edematous at the time of sacrifice. Whether this effect was due to persistent constriction of the glomerular afferent arterioles by mephentermine, promotion of the release of antidiuretic hormone by one of the drugs, or some other action is not known.

DISCUSSION

Although Iso-OMPA exhibits variable effects according to species,[3,4] it has been confirmed as a remarkably selective inactivator of BuChE of cat tissues, *in vitro* and *in vivo*. In the former situation, its selectivity was enhanced when it was used at 5° for 60 min; this is of particular value for the treatment of tissue sections of various thicknesses, since it permits more effective penetration of the inhibitor than would occur at shorter periods. The only limitations of Iso-OMPA as an inactivator *in vivo* is its failure to penetrate the blood–brain barrier effectively after doses that produce essentially total inactivation of BuChE at sites outside the central nervous system. The lack of detectable pharmacological effects, aside from possible enhancement of intestinal motility, in such circumstances is a reminder that the physiological function of BuChE is still unknown.

Astra 1397 has been used infrequently as a selective inhibitor of BuChE,[5] in contrast with its close analog, ethopropazine,[15,16] which differs from the former only in its lack of a keto-group in the side-chain. The presence of this polar group confers on Astra 1397 a much higher degree of aqueous solubility, which permitted its administration, unlike ethopropazine, in the high doses required for the protection of BuChE against inactivation by 217 AO *in vivo*. The high ratio of the concentration of Astra 1397 to that of 217 AO needed for this purpose, both *in vitro* and *in vivo*, is of the same order of magnitude as that of physostigmine to diisopropyl phosphorofluoridate required for protection of AChE against irreversible inactivation;[17] it is consistent with the relative forces involved in reversible inhibition and irreversible alkylphosphorylation.[9] In addition to being a selective inhibitor of BuChE, Astra 1397 is a muscarinic blocking agent, antagonist of nicotine convulsions, antihistaminic and local anesthetic agent;[18–20] hence, the preliminary doses of atropine given here were probably unnecessary.

While the possibility cannot be excluded that some degree of inactivation of AChE or BuChE occurred during or following homogenization in tissues taken from animals that had been treated with Iso-OMPA or 217 AO *in vivo*, it is unlikely that this occurred to a significant extent in the present study, particularly in the ganglia. Homogenization was conducted at approximately 2° in a volume of water ranging from 500 to 3000 times that of the tissue, so that any uncombined inhibitor would have been diluted immediately by a minimum of three orders of magnitude. In the histochemical evaluations conducted in parallel, which provided visual confirmation of near-total degrees of enzymatic inactivation, the dilution effect that occurred when tissue sections were first exposed to an aqueous medium was several orders of magnitude greater.

Acknowledgements—Technical assistance was provided by Miss Ashley V. Fine, Mrs. Eileen R. Hathaway and Mrs. Katherine Young. Photomicrographs were taken by Mr. Edward F. Glifort. We greatly appreciate generous gifts of Astra 1397 from Dr. Murray R. Blair, Astra Pharmaceutical Products, Inc., Worcester, Mass., Dr. Berndt Sjöberg, Astra Läkemedel AB, Stockholm, and Dr. K.-B.

Augustinsson, Stockholm. 217 AO was kindly supplied by Dr. Robert A. Lehman, Ayerst Laboratories, New York, N.Y., and mephentermine by Dr. Daniel L. Shaw, Jr., Wyeth Laboratories, Radnor, Pa.

REFERENCES

1. G. B. KOELLE, R. DAVIS and M. DEVLIN, *J. Histochem. Cytochem.* **16,** 754 (1968).
2. G. B. KOELLE, R. DAVIS and E. G. SMYRL, *Prog. Brain Res.* **34,** 371 (1971).
3. W. N. ALDRIDGE, *Biochem. J.* **53,** 62 (1953).
4. L. AUSTIN and W. K. BERRY, *Biochem. J.* **54,** 695 (1953).
5. K.-B. AUGUSTINSSON, *Scand. J. Lab. Clin. Med.* **7,** 284 (1955).
6. R. J. MCISAAC and G. B. KOELLE, *J. Pharmac. exp. Ther.* **126,** 9 (1959).
7. G. L. ELLMAN, K. D. COURTNEY, V. ANDRES, JR. and R. M. FEATHERSTONE, *Biochem. Pharmac.* **7,** 88 (1961).
8. M. P. FULTON and G. A. MOGEY, *Br. J. Pharmac. Chemother.* **9,** 138 (1954).
9. J. A. COHEN and R. A. OOSTERBAAN, in *Handbook of Experimental Pharmacology* (Ed. G. B. KOELLE), Vol. XV, pp. 299–373. Springer, Berlin (1963).
10. O. ERANKÖ, G. B. KOELLE and LIISA RÄISÄNEN, *J. Histochem. Cytochem.* **15,** 674 (1967).
11. K.-B. AUGUSTINSSON and D. NACHMANSOHN, *Science, N.Y.* **110,** 98 (1949).
12. G. B. KOELLE, *J. Pharmac. exp. Ther.* **100,** 158 (1950).
13. G. B. KOELLE, *J. Pharmac. exp. Ther.* **114,** 167 (1955).
14. B. HOLMSTEDT, *Acta physiol. scand.* **40,** 331 (1957).
15. J. J. GORDON, *Nature, Lond.* **162,** 146 (1948).
16. A. TODRICK, *Br. J. Pharmac. Chemother.* **9,** 76 (1954).
17. G. B. KOELLE, *J. Pharmac. exp. Ther.* **88,** 232 (1946).
18. R. DAHLBOM, T. EDLUND, T. EKSTRAND and A. KATZ, *Archs int. Pharmacodyn. Thér.* **90,** 241 (1952).
19. R. DAHLBOM, H. DIAMANT, T. EDLUND, T. EKSTRAND and B. HOLMSTEDT, *Acta pharmac. tox.* **9,** 163 (1953).
20. R. DAHLBOM, T. EDLUND, T. EKSTRAND and A. LOHI, *Acta pharmac. tox.* **9,** 168 (1953).