Biochemical Pharmacology, Vol. 26, pp. 1821–1823. Pergamon Press, 1977. Printed in Great Britain.

Protection against organophosphate poisoning *in vivo* and inhibition of choline-acetyltransferase *in vitro*

(*Received* 25 *January* 1977; *accepted* 10 *March* 1977)

Poisoning by organophosphates and carbamates leads to an inhibition of the enzyme acetylcholinesterase (AChE). As a consequence, acetylcholine (ACh) accumulates at the synapses and neuromuscular junctions [1]. Antidotal therapy and prophylaxis against those intoxications should be possible by either protection of AChE or its reactivation after inhibition, removal of excessive ACh from the cholinergic receptor or, at least, by reducing the amount of ACh by influencing its release or formation. The last step in the biochemical synthesis of ACh is performed by the enzyme choline-acetyltransferase (EC 2.3.1.6, ChAc). Several potent inhibitors of ChAc have been described in the literature [2–5].

In the course of our biological screening tests in mice of potential organophosphate antidotes we found the compound 4-(α-naphthylvinyl)pyridinium hydrochloride (NP) to be slightly effective against intoxications by soman [*O*-(1.2.2-trimethylpropyl)-methylphosphonylfluoride]. Because NP is known as a typical ChAc-inhibitor [3], we included several similar compounds into the screening program and, in addition, we investigated their inhibitory effect upon ChAc *in vitro*.

Materials. The compounds HH 15, HH 16 and HH 28 were gifts from Prof. I. Hagedorn, Freiburg; NP was synthesized by Dr. Reiner, Frankfurt. All other compounds were prepared in our laboratory according to the literature cited in the table. Little soluble iodides were converted into the chlorides by cation exchange on Dowex WX 50. In aqueous buffered solution, all pyridinium salts proved to be stable in the dark. Storage and application was performed under exclusion of light. When exposed to the light of a 60 W bulb (distance 30 cm, 30°), the aqueous solutions of several compounds underwent time dependent spectral changes (the half life of the hypsochromal shift of the long wavelength band is given in parenthesis): HH 13 (140 min at 355 nm), HH 14 (131 Min at 365 nm), HH 28 (115 min at 352 nm), HH 30 (261 min at 342 nm), HH 59 (350 min at 341 nm), NP (83 min at 331 nm). The spectral changes are accompanied with a decrease in inhibitory strength towards ChAc. These observations agree well with the results reported by White and Cavallito, who found the hypsochromal shift to be caused by a trans-cis conversion [6].

[1-^{14}C]Acetylcoenzyme A (spec. radioactivity 58 mCi/mmile) and [1-^{14}C]acetylcholine chloride (spec. radioactivity 17.6 mCi/m-mole) were purchased from Amersham Buchler, Braunschweig. The radioactive acetyl-CoA was diluted with unlabelled acetyl-CoA (80% pure, Boehringer, Mannheim) to give a solution with the spec. radioactivity of 19.2 mCi/m-mole.

Homogenates from rat brain were used as a source of ChAc: In a Potter–Elvehjem glass/teflon homogenisator rat brain was homogenized at 0° in a 2-fold volume (w/v) of 0.25 M sodiumphosphate buffer, pH 7.4, containing 0.2% Triton X 100; 250 μl portions were stored at −30° and used without further purification. At −30° the enzymatic activity remained nearly constant over a period of three months.

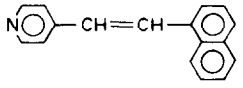
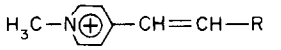
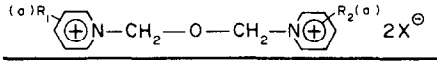
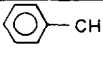
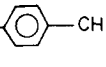
Protective activity against soman in vivo. To evaluate the protective activity we determined the ED_{50} of the pyridinium compounds against a LD_{95} of soman. The pyridinium salt was administered i.m. (aq. solution containing 0.9% NaCl) to female NMRI-mice (av. body weight 23 g). Five min later a LD_{95} of soman (0.280 mg/kg in aq. solution containing 0.9% NaCl and 2% ethanol) was given s.c. Groups of eight mice were used in each experiment. Based upon the number of deaths occurring within the following 24 hr, the ED_{50} was evaluated according to Litchfield and Wilcoxon [7].

Inhibition of ChAc in vitro. The inhibition was performed by mixing 3 μl of the pyridinium salt solution with 2 μl rat brain homogenate and incubating 1 min at 37°. The enzymatic activity was determined according to Fonnum [8] with some slight modifications; the enzymatic reaction was started by addition of 5 μl substrate. The final concentrations in the incubation mixture were: 8.75 mM choline bromide, 0.093 mM acetyl-CoA, 240 mM NaCl, 80 mM physostigmine sulfate and inhibitor depending upon the inhibitory strength. After 5 min the reaction was stopped by dilution with 5 ml 0.11 mM acetylcholine chloride in 10 mM sodium phosphate buffer pH 7.4 and 2 ml of a 0.5% (w/v) solution of Kalignost (K-tetraphenylborate) in abs. acetonitrile. The acetylcholine-tetraphenylborate was separated from the aqueous reaction mixture by shaking with 10 ml Instafluor® (Packard Instruments). The content of labelled acetylcholine in the organic layer was measured in a scintillation counter. Control runs were performed in the same way using 3 μl water instead of the inhibitor solution. The percentage of inhibition was plotted versus inhibitor concentration. The inhibitor concentration producing 50 per cent inhibition (I_{50}) was read from this graph.

Control experiments. Several preliminary checks served to ensure a proper function of the experimental arrangement: it was established that (1) the inhibition of AChE by physostigmine was complete, (2) a linear calibration curve for ACh was obtained, (3) the additional quench, caused by the pyridinium salts, was—if any—within the experimental error, (4) the pyridinium salts did not impair the extraction yield for ACh, (5) the time course of the enzymatic reaction during the incubation period was linear and (6) the interference by side reactions between acetyl-CoA and the pyridinium salts could be excluded. This possibility had to be taken into account, because, for instance, *N*-alkylpyridiniumoximes are known to react with acetyl-CoA giving acetyloximes [9]. Appropriate control experiments were made by mixing the pyridinium salts with acetyl-CoA (0.1 M both in 80 mM phosphate buffer, pH 7.4) and measuring the CoA formed after different time intervals. CoA was determined with Ellman's reagent (5,5'dithio-bis-2-nitrobenzoate) photometrically. The results indicated, that during the 5 min incubation period less than 1% acetyl-CoA will be lost by this side reaction.

Results. In vivo, the compounds HH 13 and HH 14 proved to be the best protectors against the intoxication by the organophosphate. With these pyridinium oximes

Table 1. Protection against soman *in vivo* and inhibition of ChAc *in vitro*

Compounds				$ED_{50} \times 10^5$ moles/kg	$I_{50} \times 10^5$ M
NP				5.3 (4.2-6.7)	6.4†
					
	R		X		
HH 30[3,10]			Cl	–	2.8†
HH 59[3,10]			Cl	–	0.42†
HH 13[10]			J	1.4 (1.2-1.7)	>100
HH 34[10]	$-CONH_2$		J	–	7.8
HH 15	-CHNOH		J	–	40 ($=I_{25}$)
					
	a	R_1	R_2	X	
HH 28	4		$=R_1$	Cl	6.0 (4.6-7.9)	38.0
HH 14[10]	4	HONCH—  —CHNOH		Cl	1.2 (0.8-1.4)	>100
HH 51[10]	4	$H_2NOC-CH=CH-$	$=R_1$	J	3.8 (3.1-4.7)	31.5
HH 48[10]	3	$H_2NOC-CH=CH-$	$=R_1$	J	4.8 (3.9-5.9)	>100
HH 16	4	HONCH—CH=CH—	$=R_1$	Cl	–	>100
HP 71[11]	4	$H_2NOC-NH-N=CH-$	$=R_1$	Cl	3.4 (2.8-4.3)	>100

* a denotes the position in the pyridine nuclei.

† for NP, HH 30, HH 59 Cavallito *et al.* [2] reported the I_{50} data 2.5, 1.5, 0.37×10^{-5} M, respectively.

The ED_{50} of pyridinium salts reduces the toxic effect of a LD_{95} of soman (s.c., mice) to a level of 50 per cent mortality; 95 per cent confidential limits in parentheses. The I_{50} of pyridinium salt reduces the original activity of rat brain ChAc by 50 per cent. No item (–) indicates that an ED_{50} (up to 1/10 of the compound's LD_{50}) could not be established.

no measurable inhibition of ChAc could be obtained. On the other hand, with the most potent ChAc inhibitors HH_{59} and HH_{30} no ED_{50} against soman could be established *in vivo*.

The inhibitory potency towards ChAc drops down upon introduction of an aldoxime group into the inhibitor molecule (HH_{30}/HH_{13}, HH_{34}/HH_{15}, HH_{28}/HH_{14}) as well as by bisquaternization (HH_{30}/HH_{28}, HH_{34}/HH_{51}, HH_{15}/HH_{16}). With regard to the protective efficiency, the ED_{50} values show the inverse relationship.

From the results with these pyridinium salts we conclude, that the protective effects against soman found *in vivo* are not caused by inhibition of ChAc.

Unlike the pyridinium salts, the nonquaternary and particularly lipophilic compound NP shows both inhibition of ChAc and protection against soman to a remarkable extent. The question as to whether here both effects are causally correlated needs further investigation.

Acknowledgements—We thank Drs. F. Fonnum and D. Malthe-Sørensen for helpful advice, Mrs. M. Krügel and I. Müller for skilled technical assistance.

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Biochemical Pharmacology, Vol. 26, pp. 1823–1824. Pergamon Press, 1977. Printed in Great Britain.

The effect of spironolactone pretreatment on the biliary excretion and renal accumulation of inorganic mercury in the rat

(*Received* 13 *December* 1976; *accepted* 18 *March* 1977)

A discrepancy has been noted in the literature on the effect of spironolactone (Sp) pretreatment on the biliary excretion of inorganic mercury [1–5]. Haddow *et al.* [1] and the authors [2, 3] reported that the biliary excretion of i.v. administered mercury in Sp pretreated rats was more than ten times higher than control rats. Conversely, Garg *et al.* [4], and more recently Klaassen [5], reported that Sp pretreatment did not produce a significant increase in mercury excretion in rats. Klaassen injected pure Sp material i.p., while Haddow *et al.* and the present authors administered oral Aldactone tablets. Because of the pharmacokinetic differences reported between the oral administration and the i.v. injection of Sp [6, 7], the difference in drug administration or the material used could be one possible cause for this discrepancy. Another difference in the procedure is the dose of mercury administered. Klaassen injected only 30 μg/100 g body weight of mercury, while Haddow *et al.* and the present authors administered a dose more than five times higher than the dose used by Klaassen. In order to find out the true cause for the discrepancy in Sp effect on the biliary excretion of mercury, we performed several tests under different experimental conditions.

Male SPF Sprague Dawley rats weighing 250–350 grams were used. Commercial Sp tablets (Aldactone A. G. D. Searle & Co., Chicago, IL) were ground into powder and suspended in distilled water or ethylene glycol. A water suspension of powdered Aldactone was given orally through a stomach tube, or injected i.p. Ethylene glycol suspension was given i.p. Pure Sp material was purchased from Sigma Chemical Company (St. Louis, MO). This Sp material was suspended in ethylene glycol or propylene glycol and was administered i.p. The Sp dose was 5 mg/100 g B.W. In control rats, only ethylene glycol or propylene glycol was administered i.p. Mercury excretion studies were performed on rats 1–2 hr after the pretreatment. Under pentobarbital anesthesia (4.5 mg/100 g i.p.), the common bile duct was cannulated (PE-10 tubing) and a saline solution of mercuric chloride containing $^{203}HgCl_2$ (RCC, Amersham, England) was injected i.v. Two different mercury doses (0.2 mg/100 g, 30 μg/100 g) were tested in separate experiments. Four 30 min cumulative bile samples were collected during the following 2 hr. The rectal temperature was maintained between 37° and 38° throughout the experiment. Thereafter, rats were exsanguinated and the liver and both kidneys removed. The recovery of the i.v. administered mercury in the bile and the mercury content in these organs were then measured for their radioactivity. The means of the treated groups were compared with the control value by Student's *t* test.

The biliary recovery of i.v. administered mercury and the mercury contents of the liver and kidneys expressed as a percent of the administered dose are summarized in Table 1.

Table 1. Biliary recovery and organ content of intravenously administered inorganic mercury in control and spironolactone pretreated rats (mean ± S.D., per cent of the injected dose)

Spironolactone pretreatment	Biliary recovery of mercury				Total recovery for 2 hr	Mercury content 2 hr after mercury injection	
	0–30 min	30–60 min	60–90 min	90–120 min		Liver	Kidneys
			Mercury dose 0.2 mg per 100 g body weight				
Control* (4)	0.32 ± 0.05	0.46 ± 0.08	0.39 ± 0.05	0.28 ± 0.03	1.45 ± 0.12	14.78 ± 0.92	34.77 ± 4.18
Oral (W-Al) (4)	5.53 ± 1.49§	5.15 ± 1.31§	2.17 ± 0.43§	1.22 ± 0.21§	13.13 ± 3.08§	13.30 ± 1.61	28.52 ± 4.00¶
IP (W-Al) (3)	3.71 ± 0.98§	3.77 ± 0.49§	1.98 ± 0.43§	1.03 ± 0.23§	10.49 ± 1.16§	22.07 ± 7.32¶	17.68 ± 1.50§
IP (EG-Al) (3)	5.26 ± 0.79§	4.11 ± 0.69§	2.36 ± 0.11§	1.26 ± 0.12§	12.99 ± 1.61§	15.25 ± 2.19	13.73 ± 3.67§
IP (EG-Sp) (4)	5.11 ± 1.02§	3.92 ± 0.80§	2.20 ± 0.33§	1.35 ± 0.85§	12.58 ± 1.39§	14.81 ± 1.39	6.58 ± 2.65§
			Mercury dose 30 μg per 100 g body weight				
Control† (3)	0.21 ± 0.03	0.29 ± 0.05	0.25 ± 0.03	0.18 ± 0.06	1.27 ± 0.59	7.89 ± 1.39	20.63 ± 3.50
IP (PG-Sp) (6)	1.92 ± 0.57§	1.54 ± 0.27§	0.87 ± 0.16§	0.54 ± 0.06§	4.87 ± 0.84§	12.41 ± 2.25¶	4.81 ± 1.43§
IP (EG-Sp) (3)	2.64 ± 0.50§	1.84 ± 0.19§	1.00 ± 0.02§	0.45 ± 0.17§	5.93 ± 0.54§	12.61 ± 0.88¶	4.16 ± 0.70§

* i.p. injection of ethylene glycol only † i.p. injection of propylene glycol only.
§ Significantly different from respective control value ($P < 0.01$).
¶ Significantly different from the control value ($P < 0.05$).
All pretreatments were done 1–2 hr prior to mercury study.
Spironolactone doses were all 5 mg/100 g body weight as Sp weight.
Number in parenthesis indicates the number of rat studied.
IP: intraperitoneal injection, Oral: oral administration, W: water suspension, EG: ethylene glycol suspension, PG: propylene glycol suspension, SP: pure spironolactone material, Al: powdered Aldactone A tablet.