partially conjugated with glycine and both the parent compound and its glycine conjugate are rapidly eliminated from the body.

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Comparative Toxicity of Acetylcholine Mustard (Methyl-2-acetoxyethyl-2′-chloroethylamine) in the Mouse and American Cockroach

John G. Clement,* Maurice Hirst, and E. Howard Colhoun

The toxicity of acetylcholine mustard has been investigated in the mouse and the American cockroach. This compound, resembling acetylcholine when dissolved in water, was expected to have high toxicity in the mouse and low toxicity in the insect. In contrast to acetylcholine, high toxicity was obtained in the cockroach with an LD_{50} approximating that of nicotine (base). Toxicity depended on the route of drug administration and the use of nonaqueous solvents. This procedure maintained the tertiary lipophilic nature of the pure compound and it may be an important factor in allowing the substance to reach a tissue target site. High toxicity was obtained in the mouse; toxicity was related to route of administration of the compound. The aziridinium ion of acetylcholine mustard was more toxic in the mouse than the parent tertiary compound. Symptoms of toxicity and the lack of cholinergic stimulation are discussed for both species of experimental animal. The mechanism of action of acetylcholine mustard in the mouse and insect is unknown.

Hanby and Rydon (1947) first synthesized methyl-2-acetoxyethyl-2′-chloroethylamine subsequently termed acetylcholine mustard (AChM) by Hudgins and Stubbins (1971). The toxicity of AChM in mice was investigated by Anslow *et al.* (1947) with a reported LD_{50} of 36.5 mg/kg following intravenous injection in propylene glycol. More recent work of Hudgins and Stubbins (1972), Hirst and Jackson (1972), and Robinson *et al.* (1974) has revealed muscarinic activity of the aziridinium ion of AChM which is formed in aqueous medium. Nicotinic activity of this ion was greater than acetylcholine (ACh), when tested on the frog rectus abdominis muscle and chick biventer cervicus muscle (Jackson, 1972). Although suggested by Hudgins and Stubbins (1972) and Robinson *et al.* (1974), the alkylation of receptor anions was not an obvious property of the aziridinium ion (Hirst and Jackson, 1972). Thus it appears unlikely that cytotoxicity is the prime mode of action of AChM in animals.

The preliminary data of Clement *et al.* (1973) showed that toxicity of AChM in the American Cockroach, *Periplaneta americana*, depended on injection or topical application of AChM as the tertiary base. Injection of AChM in nonaqueous media enhanced the toxicity of AChM. It is not clear from the results of Anslow *et al.* (1947) whether the toxicity of AChM in mice was dependent on the use of propylene glycol as the solvent system or indeed if the aziridinium ion was the toxic component. This problem has been investigated in the mouse and data are given for the actions of AChM in the cockroach in which ACh has no known toxicity (Colhoun, 1963).

MATERIALS AND METHODS

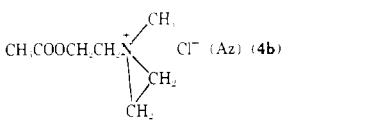
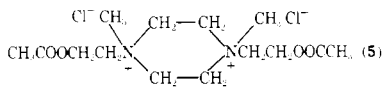
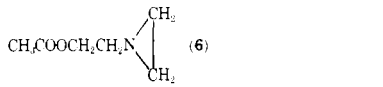
Male mice weighing 25–30 g obtained from Bio-Breeding Laboratories, Ottawa, Ontario, were used for toxicity studies. The mice supplied with water and Purina pellets were acclimated in the animal rearing laboratories for 48 hr before use in the toxicological experiments. The test substances, dissolved in physiological saline, were injected intraperitoneally (IP) or intravenously (IV) into the tail vein. Eight to ten mice were used for each experiment. Individual mice were marked and those given the same dose of drug were held together in a colony cage until the termination of the experiment. Respiratory arrest was the criterion for death in the mouse.

Male American cockroaches supplied by the Department of Zoology, or by the Research Institute, Canada Department of Agriculture, University of Western Ontario, London, Canada, were used for toxicity studies on a species of insect. The cockroaches were acclimated in the laboratory for 24 hr within glass jars containing water and Purina chow. A layer of vaseline was smeared along the top of the jar to prevent escape of the cockroaches. Similar jars were used for the insects following exposure to various drugs. Ten cockroaches were used for each experiment and all experiments were replicated at least three times. Compounds, dissolved in water or in an organic solvent, were applied topically to the dorsal abdomen of the cockroach. Acetone was used as a water-free solvent by drying it with a molecular sieve, Type 4A, B.D.H. Some of these compounds were injected intraabdominally (IAb), with a number 30 needle, between the fourth and fifth ventral abdominal sclerites. The needle was inserted at a shallow angle and allowed to penetrate approximately 0.25 in. All drugs were injected in a fluid volume of 2 μl. For control purposes the solvents were injected or applied topically in the same fluid volume. In the American cockroach the inability to stand was termed knockdown. Prolongation of knockdown to 48 hr was recorded as death. LD_{50} estimations were made according to Miller and Tainter (1944).

Precursors, metabolites, and congeners of AChM are listed below. They were prepared according to the method

Faculty of Medicine, Department of Pharmacology, University of Western Ontario, London, Canada.

Table I. Toxicity of AChM and Congeners Injected Intraabdominally in Water into the American Cockroach

Compound	Dose, μg/roach	Toxicity
$HOCH_2CH_2N(CH_3)CH_2CH_2OH$ (1)[a]	800	Nil
$CH_3COOCH_2CH_2N(CH_3)CH_2CH_2OH$ (2)	800	Nil
$CH_3COOCH_2CH_2N(CH_3)CH_2CH_2OOCCH_3$ (3)	800	20%[b]
$CH_3COOCH_2CH_2N(CH_3)CH_2CH_2Cl$ (AChM) (4a)	300.0 ± 12.7	LD_{50}
 Cl^- (Az) (4b)	960.0 ± 22.0	LD_{50}
 (5)	800	Nil
 (6)	625.0 ± 25.0	LD_{50}

[a] Numbers in parentheses correspond to compounds listed under Materials and Methods. [b] Percent mortality.

of Jackson and Hirst (1972). The numbers in parentheses refer to the listing of chemicals in Table I: 2-(methylamino)ethanol (Aldrich Chemical Co.) (**1**); methyl-2-acetoxyethyl-2′-hydroxyethylamine (**2**); methyldi-2-acetoxyethylamine (**3**); methyl-2-acetoxyethyl-2′-chloroethylamine (**4a**); *N*-methyl-*N*-(2-acetoxyethyl)aziridinium (**4b**); *N,N*′-dimethyl-*N,N*′-di-(2-acetoxyethyl)piperazinium dichloride (**5**); 1-(2-acetoxyethyl)aziridine (**6**). The purity of AChM was repeatedly monitored by gas–liquid chromatography and the samples used in the investigations were consistently 95% + AChM. All concentrations of AChM refer to the free base.

Throughout this paper "AChM" refers to the tertiary form of methyl-2-acetoxyethyl-2′-chloroethylamine (**4a**) and "aziridinium ion" (Az) refers to the cyclic quaternary species, *N*-methyl-*N*-(2-acetoxyethyl)aziridinium (**4b**) which forms in an aqueous medium. AChM when dissolved in water forms a maximum concentration of about 70% aziridinium ion in 30 min at 25° (Hirst and Jackson, 1972).

RESULTS

The results given in Table I show the toxicity of AChM and congeners when injected intraabdominally in water into the American cockroach. The compounds given in Table I are listed in order of organic synthesis with AChM being compound **4**. Compounds **5** and **2** can form through spontaneous decomposition and are minor contaminants of AChM. The aziridine compound, **6**, which is not present in the synthesis or degradative pathway, was also checked for toxicity.

It is clearly evident from the results given in Table I that AChM (**4**) was toxic to the American cockroach. Low toxicity was found with compound **3**, the immediate precursor of AChM, and some toxicity was found with the aziridine, compound **6**. Of the compounds tested in Table I AChM most closely resembles ACh which is not toxic to insects (Colhoun, 1963). AChM is a tertiary base with a pK_a = 6–6.5 (Hirst and Jackson, 1972) and thus differs in lipophilic characteristics from ACh which is a quaternary ion. Lipid solubility may be an important factor contributing to the toxicity of AChM.

The results given in Table II show the toxicity of AChM, given by two routes of administration and in different solvents to the American cockroach. Nicotine (base) was used for comparative purposes. The toxicity of AChM dissolved in water or acetone was greater when injected intraabdominally than the compound in the same solvents applied topically to the dorsal abdomen. AChM dissolved in water was essentially nontoxic when applied topically to the cockroach (LD_{50} > 1200 μg) whereas AChM applied topically in acetone had an LD_{50} close to the value obtained by intraabdominal injection. The significance of organic solvents in determining the toxicity of AChM in the cockroach is further evident when mortalities are compared using propylene glycol, acetone, and water as solvents. Injected AChM in propylene glycol had the highest toxicity followed by AChM in acetone and then in water. The variable toxicity of AChM depending upon the route of administration and the solvent system is probably due to preservation of the molecule as a lipid-soluble tertiary amine. Depending upon time and ambient temperature noncyclized or tertiary AChM forms a quaternary ion (cyclized) in water. The tertiary amine would have greater potential in transport through biological barriers and would be more able to reach a tissue target site of action.

Toxicity of AChM was enhanced when cockroaches injected with AChM were pretreated with a sublethal dose of physostigmine (0.63 μg/cockroach). This enhancement of toxicity suggests that a site of action of AChM may be in the central nervous system of the cockroach which contains large amounts of acetylcholinesterase (AChE) (Colhoun, 1959). Following formation of the aziridinium ion, by allowing AChM to stand in water for 30 min at 25°, the substance was injected into cockroaches. Negligible toxicity was found and this result is in striking contrast to AChM injected immediately in water, acetone, or propylene glycol. As shown in Table II ACh was not toxic to the cockroach.

Although AChM, as the aziridinium ion, has known potent nicotinic and muscarinic properties the toxicity of the compound given to the cockroach as the tertiary base was not manifested by characteristic symptoms of cholinergic intoxication. After a lethal dose of AChM cockroaches showed some transitory stimulation which was more evident when acetone was used as the organic solvent. The period between application of AChM and knockdown was characterized by stiltlike movements of legs, and greater than normal walking activity. Shortly before knockdown, cockroaches were unable to cling onto the side of the glass container and fell to the floor. Once in this position they had difficulty righting themselves. Symptoms of weakness were progressive and this type of weakness prompted use of the expression "insect myasthenia gravis." Time to knockdown was approximately 2 hr at the LD_{50} level and there was no evidence of recovery once the cockroaches were prostrate. In contrast, nicotine-treated cockroaches showed immediate symptoms of hyperactivity with convulsions preceding knockdown. Knockdown occurred within minutes at the LD_{50} level of poisoning and in the prostrate stage following knockdown, legs and other appendages exhibited fine high frequency tremors. Despite the rapidity of onset of symptoms with nicotine, which was similar even at low doses of the substance, toxicity depended on recovery from the initial dose. At low doses of nicotine, recovery of treated cockroaches required several hours; at higher doses recovery was longer and unless the insects recovered within 24 hr, toxicity was irreversible.

The toxicity of AChM and the aziridinium ion injected IV or IP into male mice is given in Table III. The aziridinium ion injected IV gave the greatest toxicity with an LD_{50} of 4.6 ± 0.12 mg/kg. The toxicity of AChM given by the same route was much lower (9.15 ± 0.51 mg/kg). (AChM was dissolved in saline and injected immediately into a mouse. All injections were complete within 5 min. During this time formation of the aziridinium ion would

Table II. Toxicity of AChM Given by Several Routes of Administration Using Various Solvents in the American Cockroach

Compound	Solvent	Administration route	LD_{50}, μg/roach
ACh	H_2O	IAb[a]	>2000
AChM	H_2O	Topical	>1200
AChM	H_2O[c]	IAb	300.0 ± 12.7[b]
Aziridinium ion	H_2O[d]	IAb	960.0 ± 22.0
AChM	Acetone	IAb	250.0 ± 104.0
AChM (eserine pretreated)	Acetone	IAb	105.0 ± 10.1
AChM	Acetone	Topical	315.0 ± 7.5
AChM	Propylene glycol	IAb	204.0 ± 7.9
Nicotine (base)	Acetone	Topical	185.0 ± 17.0

[a] IAb, intraabdominal injection. [b] LD_{50} ± SD. [c] AChM was dissolved in water and injected IAb. Time from dissolving AChM in H_2O and completion of injection was less than 5 min. [d] AChM was dissolved in water and allowed to stand at room temperature for 45 min before injection.

be minimal.) Toxicity of AChM or the aziridinium ion was also dependent on the route of administration. Comparison of the LD_{50} values for each route of administration clearly illustrates this conclusion. Time to death in treated mice was also related to the route of drug administration. The results given in Table III suggest that the LD_{50} value of AChM or its aziridinium ion is much lower than the LD_{50} value (36.5 mg/kg) reported by Anslow *et al.* (1947). The toxicities of the AChM precursor, compound **2**, also a contaminant, and compound **5**, a decomposition product of the aziridinium ion, were tested in the mouse. As found with the American cockroach, toxicity of these compounds in the mouse was low. The aziridine compound 6 had low toxicity in the mouse with an LD_{50} close to 140.0 mg/kg.

Upon IV administration of an LD_{50} dose of the aziridinium ion in the mouse, poisoning was characterized by occasional transient convulsions, muscle fasiculations, and apnea which increased in intensity with larger doses. Within 30–60 sec the mouse then appeared to recover. Approximately 5–7 min later depending on the route of administration, muscular weakness and prostration became evident along with bronchial congestion and depressed and labored respiration. Twitches developed at approximately 10 min after injection of the ion followed by convulsions; death ensued usually within a few minutes due to apparent respiratory paralysis.

To obtain toxicity with IP injections of aziridinium ion, larger concentrations were used and only then did typical signs of cholinergic stimulation appear, such as lacrimation, diarrhea, and increased salivation. However, no stimulation was noted immediately following IP injection. This result is in contrast to the initial symptoms observed with IV injections of the mice presumably due in part to a bolus effect of the latter. Symptomology preceding death was similar to that observed by IV administration. Exophthalamus was observed at high concentrations of ion (10 mg/kg or greater). With AChM some hypopnea and transient convulsions were evident following IV injections. Death occurred approximately 11 min later (Table III) with symptoms similar to those described above for IP and IV routes.

Mice surviving a dose of AChM or the aziridinium ion were noted to be depressed for 24 hr. They were anorexic and hypotonic, and the palpebral opening was reduced to half. Within a further 24 hr these symptoms disappeared and the mice appeared to be fully recovered.

DISCUSSION

It is clearly evident from the results presented in this paper that AChM is highly toxic to both the American cockroach and the mouse. Furthermore, toxicity in each species of test animals is not characteristic of poisoning related to stimulation of the cholinergic nervous system. These facts were outlined in the description of symptoms of intoxication observed in poisoned mice and cockroaches. Toxicity is also dependent on the use of the tertiary AChM or the quaternary aziridinium ion and on the route of administration.

Table III. Toxicity of AChM, Aziridinium Ion, and Hydrolysis Products to Mice

Compound	Route	LD_{50}, mg/kg	Time to death at LD_{100}, min
Aziridinium ion	IP	12.8 ± 2.0[a]	22.5
Aziridinium ion	IV	4.6 ± 0.12	12.0
AChM	IV	9.15 ± 0.51	11.5
Dimer (**5**)[b]	IV	>100.0	
AcOH (**2**)	IV	>100.0	
ACh	IV	21.0 ± 3.9	<1.0

[a] LD_{50} ± SD. [b] Numbers in parentheses correspond to compounds listed in Table I.

In the insect, AChM and not the aziridinium ion was the toxic form of the molecule upon administration. The toxicity of this lipophilic tertiary amine was enhanced by the use of acetone for topical application and acetone or propylene glycol for IAb injection. These factors are seen as promoting absorption through a biological membrane and slowing down formation of the aziridinium ion until it is near a tissue target site. It should be noted that the aziridinium ion closely resembles ACh in chemical structure (Hirst and Jackson, 1972) but with some change in the shape of the ammonium head. It is well known that ACh (Treherne and Smith, 1965; Lord *et al.*, 1967; Eldefrawi and O'Brien, 1967; Treherne and Pichon, 1972) can move through the nerve sheath of the cockroach and is hydrolyzed by AChE located near the periphery of the ganglia. This enzymatic action together with other possible membrane barriers found within the ganglia near synapses apparently prevents exogenous ACh from stimulating cholinergic receptors. The potential and distribution of ACh as a synaptic transmitter agent in the cockroach have been reviewed by Colhoun (1963) and the elegant experiments of Shankland *et al.* (1971) clearly show the neurotransmitter role of ACh in the cockroach sixth abdominal ganglion. AChM would be expected to move readily through the perineurium of the cockroach nerve cord and have the potential to reach synaptic transmitter sites before complete formation of the aziridinium ion. Unpublished data of Clement and Colhoun have shown

that cockroach AChE can hydrolyze the aziridinium ion of AChM at a rate similar to ACh. This may help to explain greater toxicity of AChM in physostigmine treated cockroaches. Should the prime action of AChM be in the nervous system of the cockroach then the potential for hydrolysis of the aziridinium ion must be taken into consideration, along with the lack of cholinergic symptoms, to explain toxicity. The detailed results for the cockroach confirm the preliminary data of Jackson (1972) for the housefly that AChM is toxic in insects.

In saline, AChM was found to be more toxic to the mouse than previously reported by Anslow *et al.* (1947) where the substance was injected IV in propylene glycol. This result may be due to the degree of purity of AChM used by Anslow *et al.* (1947) and/or the inability of AChM to readily form the potentially toxic aziridinium ion in propylene glycol. In the present experiments, the aziridinium ion was found to be more toxic than AChM given by the same route of administration. The ion proved to be more toxic injected IV than given by the IP route of administration. The latter result is not surprising as the quaternary nature of the aziridinium ion would preclude rapid absorption from the peritoneal cavity. In addition the unstable ion would probably be subjected to enterohepatic circulation and some degree of degradation might occur in the liver.

The toxicity of the aziridinium ion in the mouse, in contrast to the toxicity of AChM in the cockroach, would seem realistic as the potent muscarinic (Hirst and Jackson, 1972; Hudgins and Stubbins, 1972; Robinson *et al.*, 1974) and nicotinic (Jackson, 1972) aziridinium ion would readily gain access to peripheral cholinergic receptor sites. There is no evidence of cholinergic neuromuscular transmission in the insect (Colhoun, 1963). Lower toxicity of AChM in the mouse, in contrast to the aziridinium ion administered by the same route, would be dependent on rate and degree of ion formation in plasma and bioavailability of this parent molecule or ion following administration.

However, no evidence of cholinergic excitation was readily discernible at a lethal dose of the ion, or AChM in the mouse. This anomaly may have explanation in unpublished evidence of Clement and Colhoun, that erythrocyte AChE can readily hydrolyze the aziridinium ion. A metabolite of the aziridinium ion may be the toxic agent. The finding of this metabolite in the insect and mouse might reveal the causal agent for the lethargy accompanying toxicity in both animal species. This matter is under investigation.

In conclusion it is clearly evident from the experimental data presented in this article that AChM has little potential as an insecticide. However, use of the molecule has been of value in reinforcing existing information in literature that highly ionized and nonlipid soluble chemical substances do not readily gain access to target sites in the insect central nervous system. Should AChM be metabolized *in vivo* to a choline analog by hydrolysis of the ester bond it is possible that the resultant choline mustard (Az ion), by competing with choline at a presynaptic site, may have pharmacological and toxicological merit yet to be realized.

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