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EFFECTS OF 2-PYRIDINIUM ALDOXINE METHOCHLORIDE AND ATROPINE IN RELATION TO ELEVATION OF BLOOD pH IN SOMAN-POISONED DOGS

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Abstract—The effects of various chemical treatments on the respiratory and cardiovascular deficits caused by poisoning dogs with pinacolyl methylphosphonofluoridate (soman) and on survival of the dogs were studied. A comparison was made between treatment of the poisoned dogs with atropine alone and with atropine plus 2-pyridinium aldoxime methochloride (PAM). Intravenous injection of 1 mg/kg of atropine and 104 mg/kg of PAM within 2 min after poisoning dogs with 30 μg/kg of soman i.v. (approximately 3 times the LD50) counteracted the respiratory and cardiovascular collapse produced by soman and saved 12 of 13 animals. Phenoxybenzamine, 0·5 mg/kg, injected i.v. immediately after the onset of hypertension resulting from treatment of soman-poisoned dogs with atropine and PAM affected a more rapid return to normal blood pressure levels without impairing survival. The half-time for loss of reactivatability of soman-inhibited red blood cell acetylcholinesterase (RBC (AChE) *in vivo* was 5·55 min at pH 7·4 and 12·50 min at pH 7·8 (achieved by pretreatment of the dogs with Tris buffer. At the higher pH, administration of PAM could be delayed for 20 min after poisoning with 50 μg/kg of soman, and 23·4 per cent of the inhibited RBC ChE was reactivated and 80 per cent of the dogs were saved. When the blood was at physiological pH, dogs poisoned with soman and given PAM 20 min later showed little or no recovery of inhibited RBC AChE and complete mortality.

THE INHIBITION of acetylcholinesterase (AChE) by organophosphate compounds involves a phosphorylation of the enzyme at the active site.[1] Displacement of the organophosphate group from the inhibited enzyme can usually be achieved by reaction with nucleophilic compounds such as oximes, resulting in reactivation of the enzyme.[2,3] Without addition of oximes, the inhibited enzyme becomes progressively resistant to reactivation, a process designated as "aging".[4–6] The conversion of horse serum butyrocholinesterase inactivated by diisopropylphosphorofluoridate into a non-reactivatable state was shown by Berends *et al.*[7] to be closely correlated with the loss of one of the isopropyl groups from the diisopropyl phosphorylated enzyme. Later, Loomis and Salafsky[8] reported that pinacolyl methylphophonofluoridate (soman) produced an inhibited AChE which was only slightly reactivated by oximes. Fleisher and Harris[9] showed that aging of soman-inhibited bovine erythrocyte AChE and dealkylation by loss of a pinacolyl group occur rapidly and at virtually the same rate. Rapid dealkylation of AChE inhibited by soman was also reported by Coult *et al.*[10]

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More recently, dog erythrocyte cholinesterase (CHE) inhibited by soman was shown to undergo rapid aging with a half-time approximating 5 min. Aging of the inactivated enzyme can be markedly decreased *in vitro* by pyridinium 2-aldoxime methochloride (PAM) in concentrations of $1{\cdot}25 \times 10^{-3}$M or higher, or by i.v. injection of 104 mg/kg of the same oxime into the poisoned animals.[11] This led us to study the effects of 104 mg/kg of PAM injected i.v. along with atropine on a number of physiological deficits resulting from soman poisoning in dogs.

An earlier study by Davies and Green[12] of red blood cell ChE inactivated by isopropyl methylphosphonofluoridate (sarin) *in vitro* had shown that the rate of aging could be diminished by elevation of the pH. The applicability of this observation to soman-inhibited ChE was studied by measuring the rate of aging of the inhibited enzyme at various pH levels *in vitro* and *in vivo*. In addition, the effectiveness of PAM as an adjuvant to atropine and in reactivation of inhibited RBC ChE was tested at pH 7·4 and at pH 7·8 in soman-poisoned dogs.

MATERIALS AND METHODS

Soman of 96 per cent purity was obtained from the Chemical Process Division of these laboratories. Each dose of soman was dissolved in 2 ml of a 0·9% NaCl solution and was injected i.v.

Tris buffer was made up in 0·9% NaCl so as to contain 180 g/l.

Therapeutic compounds were made up in 2 ml of 0·9% NaCl, brought to approximate neutrality with NaOH and injected intravenously.

EXPERIMENTAL AND RESULTS

A. Effects of atropine alone and of atropine plus PAM on blood pressure, respiration and heart rate in dogs poisoned with 30 μg/kg of soman. Adult mongrel dogs of both sexes were anesthetized with 30 mg/kg of sodium pentobarbital injected intravenously and more was given as needed to maintain anesthesia. The femoral artery was cannulated and the systemic blood pressure was recorded by means of a Statham strain gauge transducer on an E & M Physiograph. Electrocardiogram, respiratory rate and heart rate were continuously monitored on the same instrument by using a pair of needle electrodes placed in each side of the chest wall. After a control blood sample was obtained for measurement of the initial RBC AChE activity, all animals were given 30 μg/kg of soman (approximately 3 times the LD50) intravenously. A second blood sample was taken 1 min after poisoning. Therapeutic compounds were injected into the femoral vein at times noted below.

Surviving dogs were observed for 4–6 hr after treatment. The blood vessels were ligated and the cannulae were removed. The dogs were then placed in cages containing food and water and were kept under observation for 3 weeks. Examination of brain, muscle, heart, lungs, liver, pancreas, spleen, stomach, small and large intestine, kidneys and adrenal glands in each of 4 dogs chosen from the survivors after 3 weeks (2 from the group treated with PAM and atropine; 2 from the group treated with PAM, atropine and phenoxybenzamine) revealed no evidence of gross abnormalities.*

In 5 dogs not given atropine and PAM, respiration ceased almost immediately, while marked bradycardia and hypotension developed (Fig. 1). These animals died

* We are grateful to the Veterinary Medicine Department of Medical Laboratories, Edgewood Arsenal, Md., for the autopsy examinations.

several minutes after poisoning. These effects were similar to those noted earlier in dogs poisoned with isopropyl methylphosphonoflupridate (sarin).[13–15]

Ten dogs were given 1 mg/kg of atropine i.v. at the time of respiratory and circulatory collapse (approximately 2 min after poisoning). A brief, spontaneous increase in heart rate developed about the time of atropine injection due to "vagal escape" (shown also in untreated controls in Fig. 1) followed by a more sustained increase

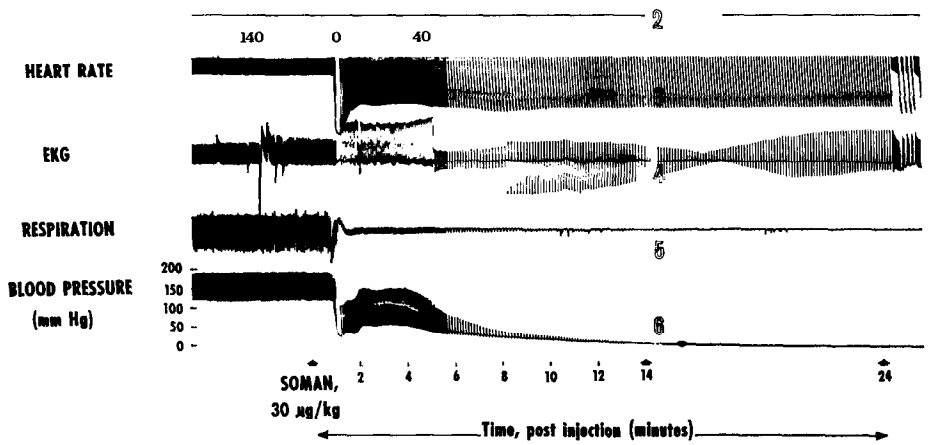

FIG. 1. Effects of poisoning with soman on heart rate, EKG, respiration and blood pressure in the dog.

in heart rate (Fig. 2). In these dogs the injection of atropine was also followed by a transient tachypnea and then by a temporary phase of hyperpnea (Fig. 2). Blood pressure rose precipitously, then gradually returned toward its original level. Subsequently, respiration weakened, then ceased, accompanied by bradycardia and a fall in blood pressure to shock levels (Fig. 2). Death ensued shortly thereafter.

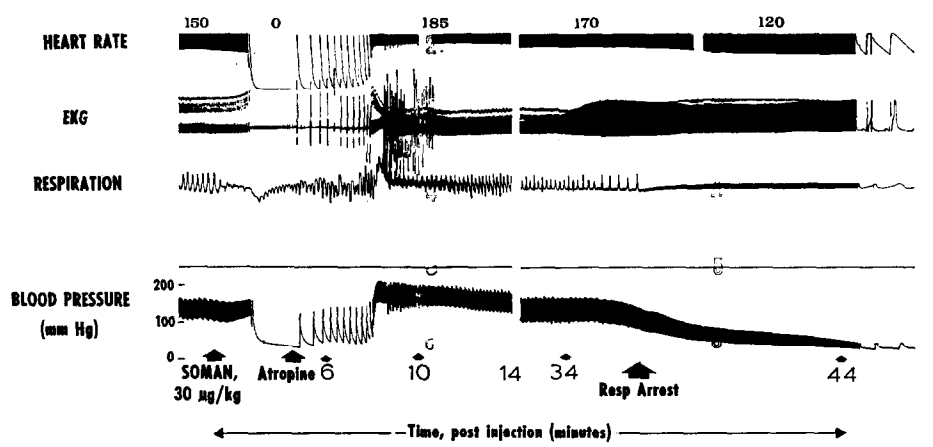

FIG. 2. Effects of treatment with atropine on soman poisoning in the dog.

In our next series, 13 dogs were given 1 mg/kg of atropine and 104 mg/kg of PAM i.v. within 2 min after poisoning with soman. Fig. 3 shows the results of the injection of atropine and PAM into the soman-poisoned dogs. Prompt elevation of the blood pressure to hypertensive levels accompanied by recovery of spontaneous respiration resulted. The blood pressure and heart rate in these animals gradually decreased to control levels within 3–4 hr. Twelve out of the 13 dogs poisoned with soman and treated with atropine and PAM in this manner survived during an observation period of 3 weeks.

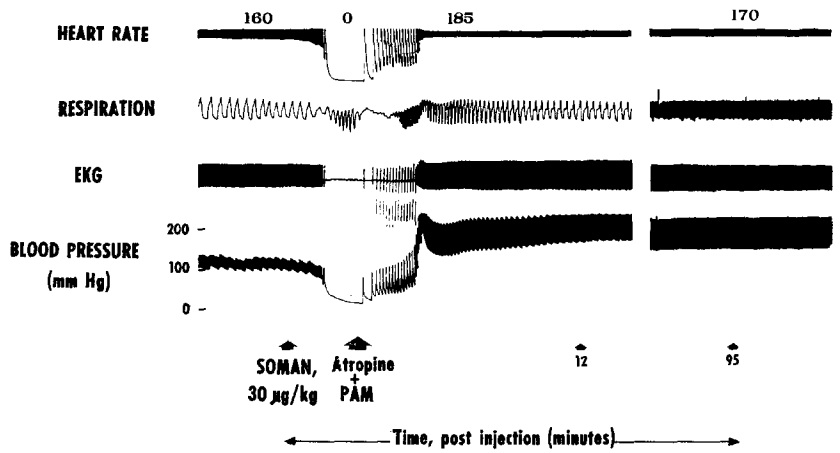

FIG. 3. Effects of treatment with atropine and PAM on soman poisoning in the dog.

Nine dogs were then given soman and treated with atropine and PAM as in the preceding group. In addition, these animals were given 0·5 mg/kg of phenoxybenzamine (dibenzyline) i.v. shortly after the onset of hypertension. Injection of phenoxybenzamine resulted in a more rapid return of blood pressure toward the normal level than was observed in the poisoned animals receiving atropine and PAM alone (Fig. 4). All 9 dogs receiving this drug regimen survived the 3-week observation period.

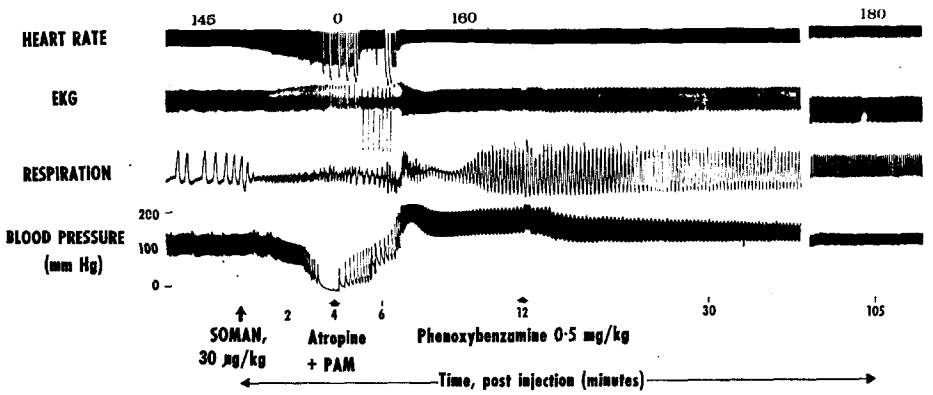

FIG. 4. Effects of treatment with atropine and PAM followed by phenoxybenzamine on soman poisoning in the dog.

RBC AChE activity in the dogs undergoing the protocols described above was measured before and after intoxication with soman and again an hour after the administration of PAM and atropine by a method reported previously.[16] Without treatment with PAM, the RBC AChE activity was less than 4 per cent of normal. In dogs receiving PAM under these conditions, a recovery of 23 $\pm$ 5·3 per cent (S.D.) was obtained.

B. Effect of elevation of pH on the rate of aging of dog RBC AChE after inhibition with soman in vitro *and* in vivo. Eight ml of heparin-treated whole blood from each of 8 dogs was added to 32 ml of ice-cold 0·9% NaCl solution adjusted to approximately pH 8·8 with 0·01 M borate buffer. The mixture was incubated with 2×10^{-7} M soman for 15 min at 0°. A preliminary study showed that almost complete inhibition with minimal aging was obtained in this manner. The sample was centrifuged at 2000 rpm for 5 min at 2°. The supernatant was discarded and the red blood cells were put into a bath at 37°; 2–3 min sufficed to equilibrate the cells to bath temperature. A 0·3-ml aliquot of packed cells was immediately transferred into 10^{-1} M PAM in 0·9% saline buffered at pH 7·6 for measurement of initial reactivatability.* A second aliquot of cells was promptly put into phosphate-buffered 0·9% NaCl without PAM to serve as an inhibition control. Rapid aging of the remaining RBC AChE was immediately started by adjusting the pH to 7·40 $\pm$ 0·05 by addition of 4 ml of 0·05M phosphate buffer in 0·9% NaCl (previously equilibrated to 37°) for each millimeter of packed inhibited red blood cells. In other studies, the pH was adjusted to 7·8. Reactivatability as a function of time of aging at both pH levels was measured as described previously.[11] Results are shown in Table 1.

TABLE 1. LOSS OF REACTIVATABILITY (AGING) OF SOMAN-INHIBITED DOG RED BLOOD CELL AChE ACTIVITY AT pH 7·4 AND 7·8

pH during aging	Type of study	Mean half-time* (min)		Rate constant* (min^{-1})	
7·4	*in vitro*	5·33	(4·80–5·70)	0·130	(0·122–0·144)
	in vivo	5·55	(5·20–5·80)	0·125	(0·119–0·133)
7·8	*in vitro*	14·05	(10·87–17·23)	0·049	(0·040–0·064)
	in vivo	12·50	(11·70–13·30)	0·057	(0·054–0·060)

* In parentheses are the 95% confidence limits.

The rate of aging of erythrocyte AChE phosphonylated by soman *in vivo* was studied in 8 dogs anesthetized with 30 mg/kg of sodium pentobarbital and given artificial respiration. The femoral artery was cannulated to facilitate rapid withdrawal of blood samples. Atropine, 1 mg/kg, was injected intravenously. Approximately 1·5 ml blood was withdrawn into a tube containing 9·5 ml of heparinized 0·9%Na Cl buffered at pH 7·6 with 0·1 M phosphate buffer and into a separate tube containing the same solution with sufficient PAM to yield a final oxime concentration of 10^{-1}M. A 50 μg/kg dose of soman (approximately 5 times the LD50) was injected via the femoral vein into each dog. Blood samples were then taken at known time intervals into the

* Measurement of the RBC AChE activity after incubation with the high concentration of PAM used required 4 cycles of washing the red blood cells with 0·9% NaCl and centrifugation in order to eliminate the anticholinesterase effect of PAM itself on the enzyme.[17]

solutions containing 10^{-1} M PAM and within 10 sec into the phosphate saline solution alone for the corresponding inhibition control. The samples were then processed as described previously.[11]

In 8 additional dogs, Tris buffer was infused into the venous circulation until a pH of 7·8 was reached. The pH was maintained at 7·8 by further infusion of Tris buffer for 20 min after poisoning. The amount of buffer required to achieve and maintain the blood pH of 7·8 for this period ranged from 300 to 360 mg/kg. The pH remained well above normal for 1–2 hr after infusion of buffer was stopped. The animals required artificial respiration during this time. When the pH of 7·8 was reached, samples of whole blood were taken into oxime and into buffer alone. Atropine, 1 mg/kg, was administered intravenously. Soman was injected and blood samples were taken as in the preceding study. The logarithm of the percentage of reactivatable RBC AChE, plotted as a function of time elapsing between injection of soman and sampling into PAM, followed first-order kinetics at each pH studied (Fig. 5). The

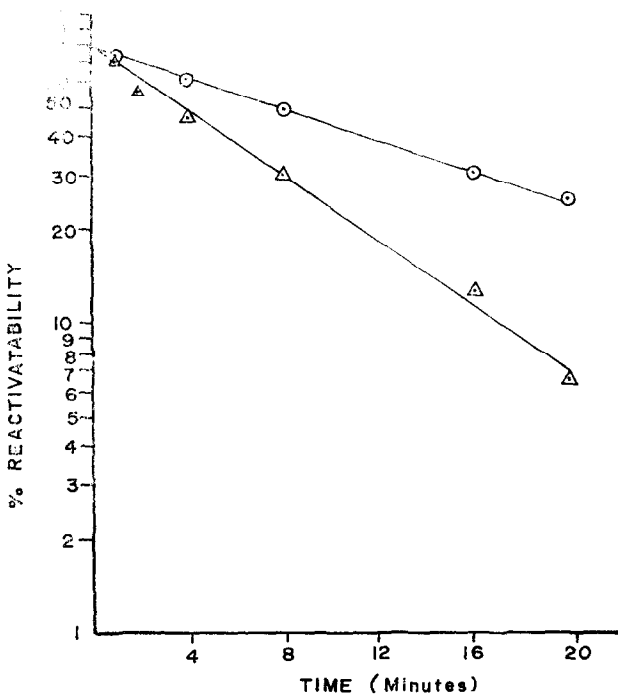

FIG. 5. Decrease in reactivatability of soman-poisoned red blood cell cholinesterase from Tris-pretreated and control dogs. △—△, pH 7·4; ○—○, pH 7·8.

reactivatability of soman-inhibited RBC AChE, as measured by incubating with 10^{-1} M PAM, was greater at pH 7·8 than at pH 7·4 under both *in vitro* and *in vivo* conditions. The mean values for the half-times of aging *in vitro* and *in vivo* $\pm$ P = 0·95 for the 8 dogs studied at each pH are shown in Table 1.

C. Effects of elevation of pH on RBC AChE activity and survival in atropinized dogs poisoned with soman and treated with PAM. The data in Fig. 5 relating decrease in reactivatability to time after injection of soman show that approximately 23 per cent

reactivation can be expected when PAM is injected 20 min after poisoning with soman, provided the pH of whole blood is maintained at 7·8. The corresponding predicted value for per cent reactivatability in control animals poisoned at pH 7·4 is 7 per cent.

These predictions were tested in dogs anesthetized with 30 mg/kg of sodium pentobarbital, infused with Tris buffer as described above and then given 1 mg/kg of atropine before the i.v. injection of 50 μg/kg of soman. Twenty min later, 104 kg/kg of PAM was injected. After 1 hr, a blood sample was withdrawn for measurement of AChE activity. Atropinized dogs were given soman, but not treated with PAM, and others governed by the same protocol without infusion of Tris buffer were included for comparison. The results are shown in Table 2.

TABLE 2. EFFECTS OF pH ON RBC AChE ACTIVITY AND SURVIVAL IN DOGS POISONED WITH SOMAN

Treatment	RBC residual AChE activity (% ± P = 0·95)	Survival ratio
Atropine + soman	5·1 ± 2·3	0/10
Tris + atropine + soman	5·1 ± 2·5	0/10
Atropine + soman + PAM	9·2 ± 4·2	0/10
Tris + atropine + soman + PAM	23·4 ± 6·7	8/10

Pretreatment of dogs with Tris buffer did not affect the inhibition of RBC ChE by soman nor did it lessen the mortality following injection of the organophosphorus compound. In the absence of Tris pretreatment, PAM injected into these dogs 20 min after poisoning did not significantly reactivate RBC AChE (Table 2). When dogs were pretreated with Tris prior to poisoning with 50 μg/kg of soman, followed by oxime injection 20 min later, 23·4 per cent recovery of the inactivated RBC AChE activity was obtained and 8 of 10 dogs survived a 48-hr observation period.

Despite pretreatment with Tris buffer and the administration of artificial respiration, all dogs that received 50 μg/kg of soman eventually showed cardiovascular collapse similar to that demonstrated in Figs. 1–4. These animals died within several hours or overnight after poisoning. Dogs given 104 mg/kg of PAM in addition to atropine developed hypertension similar to that shown in Fig. 3. Unlike the animals in the study described in section A, which received atropine plus PAM shortly after poisoning and recovered the capacity to breathe spontaneously shortly after administration of chemotherapy, the Tris-pretreated animals required artificial respiration for an additional 1–2 hr after receiving PAM plus atropine. Concurrent with a decrease in blood pH toward the normal range, the animals regained the ability to breathe spontaneously. These animals were treated in the same way as the earlier group (section A), put into cages containing food and water and observed for 48 hr.

DISCUSSION

In acute poisoning with organophosphorus anticholinesterase compounds, the reduction of RBC AChE activity has been reported to approximate that occurring in a peripheral tissue, such as the diaphragm of the poisoned animals.[11] In addition,

the reactivatability of RBC AChE activity after soman intoxication and treatment with 104 mg/kg of PAM approximates that in muscle tissue.[11] It has therefore been assumed that the effects of change in blood pH on the rate of conversion of soman-inhibited RBC AChE to a state refractory to reactivation by oximes would be representative of that occurring in peripheral tissues. Estimation of this rate of aging is important, since the principal contribution of oximes to the antagonism of intoxication by organophosphate anticholinesterase compounds is limited by the conversion of phosphorylated AChE to the nonreactivatable state. Data on the rate of aging of RBC AChE, therefore, provide useful information concerning the time during which oxime therapy would be helpful in antagonizing the harmful effects of organophosphate intoxication.

We used 104 mg/kg of PAM because this dose approximates the minimum capable of decreasing the rate of aging of RBC AChE phosphonylated by soman to a degree which provides time for significant reactivation to occur.[11] This was correlated with the survival of 21 of 22 dogs challenged with 3 times the LD50 dose of soman (section A).

Elevation of the pH of whole blood to 7·8 prior to the injection of soman slowed aging to less than half the rate prevailing in soman-poisoned controls not pretreated with Tris buffer (Table 1; Fig. 5). This permitted the injection of PAM to be delayed for 20 min after poisoning yet resulted in 23·4 per cent reactivation of inhibited RBC AChE activity. This closely compares with the value obtained in soman-poisoned dogs treated by the protocols in section A and given the same dose of PAM about 2 min after poisoning. The animals pretreated with Tris buffer also showed 80 per cent survival after a challenge with 5 times the LD50 dose of soman followed by PAM injection 20 min later in contrast to no survival in controls receiving atropine and delayed PAM without preinfusion with Tris buffer (Table 2).

Extrapolation of the curves in Fig. 5 to zero time showed only 70 per cent reactivatability. This is explained by our earlier observation that some aging of phosphonylated but not yet reactivated AChE will occur during the time required for the oxime to dephosphorylate the inhibited AChE.[9,18]

It should be pointed out that 104 mg/kg of PAM is far in excess of the amount used in conventional therapy* and produces a prolonged hypertension in animals.[19,20] The pressor effect of the oxime is thought to be mediated through an increase in the levels of endogenous norepinephrine.[21] The observation by Zarro and Di Palma[21] of the antagonism by phenoxybenzamine of the pressor effects of PAM led us to use this adrenergic blocking agent to lower the blood pressure of PAM-treated dogs to normotensive levels more rapidly. We obtained this reduction with a dose of 0·5 mg/kg of phenoxybenzamine injected intravenously after PAM administration (Fig. 4).

Intravenous injection of 1 mg/kg of atropine and 104 mg/kg of PAM within 2 min after poisoning dogs with 30 μg/kg of soman (approximately 3 times the LD50) counteracted the respiratory and cardiovascular collapse produced by soman and saved 12 of 13 animals.

Phenoxybenzamine, 0·5 mg/kg, injected i.v. immediately after the onset of hypertension resulting from treatment of soman-poisoned dogs with atropine and PAM

* Since the LD50 of PAM inm an is unknown, it should be emphasized that the dose of 104 mg/kg, which is in excess of 50 per cent of the i.v. LD50 in unanesthetized dogs (Crook, J. W. and P. Cresthull unpublished observations), is in no way recommended in cases of human poisoning by anticholinesterase compounds.

effected a more rapid return to normal blood pressure levels without impairing survival.

The half-time for loss of reactivatability of soman-inhibited RBC AChE *in vivo* was 5·55 min at pH 7·4 and 12·50 min at pH 7·8 (achieved by pretreatment with Tris buffer). At the higher pH, PAM administration could be delayed for 20 min after poisoning with 50 μg/kg of soman, 23·4 per cent of the inhibited RBC AChE was reactivated and 80 per cent of the dogs were saved. In the absence of pretreatment with Tris buffer, dogs poisoned with the same dose of soman and given PAM 20 min later showed little or no recovery of inhibited RBC AChE and complete mortality.

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