Brit. J. Pharmacol. (1957), 12, 382.

THE ASSOCIATION OF BLOOD CHOLINESTERASE LEVELS WITH THE SUSCEPTIBILITY OF ANIMALS TO SARIN AND ETHYL PYROPHOSPHATE POISONING

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(RECEIVED MAY 29, 1957)

An association between the blood cholinesterase (ChE) levels and the toxicity of sarin and ethyl pyrophosphate has been established. This has been demonstrated in two types of experiment. In the first, guinea-pigs were given a non-lethal dose of sarin (*iso*propyl methylphosphonofluoridate) which reduced the blood ChE to 20% of normal, and at intervals, as the ChE level of the blood gradually recovered, separate batches were given a second dose of the same size. A comparison was then made between the blood ChE levels immediately prior to the injection of the second dose and the mortality rate. In the second, a relatively small dose of sarin or ethyl pyrophosphate was given daily to rabbits until the enzyme value fell to a steady level. LD50 values were then determined on such groups and compared with those found in saline treated ones. In both guinea-pigs and rabbits it has been shown that a depression of blood ChE to below 40% indicated an increased toxicity of sarin and ethyl pyrophosphate to these species. The approximate quantitative relation appears to be that susceptibility is increased in the ratio 1.5 when the blood ChE is reduced by half.

Toxic manifestations in anticholinesterase poisoning are directly related to the inhibition of the enzyme in the central nervous system, the parasympathetic nervous system and the neuromuscular junction (Koelle and Gilman, 1949). The level of cholinesterase (ChE) activity in the blood gives no indication of the extent of inhibition at these sites, consequently there is no close relationship between the variation of blood ChE and the onset of symptoms. Thus it is difficult to assess the precise significance of blood ChE levels in anti-ChE poisoning. Marchand (1952), however, has stated that ChE testing of the red cells and plasma appears to be a specific and reliable method of establishing or excluding significant intoxication by organo-phosphorus insecticides or war gases.

Davies (1952) pointed out that ChE estimations have been of diagnostic value in individual cases of poisoning in which the symptoms have been ambiguous and he also drew attention to the importance of low ChE values as an objective index of inadequate safety precautions or faulty handling of such compounds.

Fawley, Hagen and Fitzhugh (1952) showed that while plasma and erythrocyte ChE measurements appear to be of value in confirming a suspected acute exposure to anti-ChE compounds, they offer little help in evaluating the degree of exposure and severity of symptoms. In chronic exposures, however, ChE measurements may be of considerable value.

One aspect of the importance of blood ChE levels has not been adequately investigated. The organo-phosphorus insecticides inactivate the enzyme through the formation of a stable phosphorylated ChE, which varies with the compound (Burgen, 1949; Aldridge, 1954). The return of ChE activity to both red cells and plasma is thus very slow and it is therefore of considerable importance to determine whether the low level of the blood enzyme under these conditions is indicative of a state of increased susceptibility to subsequent doses of anti-ChE.

This paper reports the results of experiments in which the blood ChE level of mammals, lowered either by sarin (*iso*propyl methylphosphonofluoridate) or ethyl pyrophosphate, was related to

susceptibility to further treatment with the anticholinesterase used. In both guinea-pigs and rabbits we have found that a depression of blood ChE activity to below 40% was followed by an increased toxicity of sarin and ethyl pyrophosphate to these species.

Materials and Methods

Guinea-pigs and rabbits were used, but since the experiments were carried out over a long period (12 to 18 months) no rigid standardization of strain or sex was possible although all experiments were performed on adult animals.

Two anti-ChE agents were used, sarin and ethyl pyrophosphate. Both were prepared in this establishment and were more than 95% pure.

Guinea-pigs were injected subcutaneously and rabbits intravenously.

ChE determinations were carried out upon whole blood. Two methods of analysis were employed. In the earlier experiments, the Warburg manometric technique (Callaway, Davies, and Rutland, 1951) was used, but in later work the convenience of the electrometric method (Michel, 1949) made it the procedure of choice. Acetylcholine was used throughout as the substrate.

The unit of enzyme activity varied with the method of analysis used and the species under investigation, but since they were kept constant in any given experiment and the results were compared only with those obtained in the same experiment, these differences in units were unimportant. For clarity, however, the units of activity are defined in the results section appropriate to the experiment.

Results

Guinea-pigs

In the first, a batch of 100 animals was taken and from this eight were randomly chosen for the evaluation of their normal whole blood ChE. Each guinea-pig in the batch of 100 was then injected with 0.03 mg./kg. sarin, a dose which preliminary experiments had shown would produce a substantial reduction in ChE level but little or no mortality. In fact, no guinea-pigs died at this dose level and blood taken from the eight animals used for determination of pre-exposure ChE levels showed an average reduction to 23% of normal. These eight, together with an additional four, were given 0.03 mg./kg. sarin two hours after the first injection. All 12 died within a few minutes and blood obtained by heart puncture from the original eight showed that the ChE level had been reduced to an average of 5% of normal.

Twenty-four hours later a further 12 animals from the original batch were selected and blood samples taken. They were injected immediately afterwards with another dose of 0.03 mg/kg. sarin. Two hours later, or immediately after death, blood samples were again taken. A similar procedure was adopted with subsequent batches of 12 animals on the third, fourth, fifth, eighth, tenth and eleventh days. Thus for each of these batches, ChE activities were obtained before and after the second injection together with mortality records.

This experiment was repeated about one year later using 42 guinea-pigs. Batches of 6 were taken successively, the doses of sarin being the same as in the first series.

Table I

THE RELATION BETWEEN BLOOD CHOLINESTERASE ACTIVITY LEVEL AND MORTALITY IN GUINEA-PIGS

The dose of sarin was 0.03 mg./kg. given in a 1/1,000 dilution of saline. The activity, measured by the Warburg manometric method, is expressed as % of the mean pre-exposure value.

Day	Expt. 1			Expt. 2		
	Mean ChE Activity		Mortality	Mean ChE Activity		Mortality
	Before Injection	After Injection		Before Injection	After Injection	
0 ..	100	23	0/100	100	16	0/42
2 hr. ..	23	5	12/12	16	7	5/6
1 day ..	36	9	8/10	37	6	6/6
2 days ..	42	13	4/12	41	8	2/6
3 ,, ..	56	21	1/11	47	12	1/6
4 ,, ..	61	11	1/12	58	10	0/6
7 ,, ..	73	14	2/12	73	18	0/6
9 ,, ..	73	16	0/12			
10 ,, ..	78	19	0/12	78	21	0/6

In both experiments (Table I) the initial dose of sarin reduced the blood ChE activity to about 20% of its normal value without producing any mortality. Two hours later a second dose of the same size reduced it to about 6% of normal and killed a total of 17 out of 18 animals in the combined experiments. Regeneration of the blood ChE activity occurred exponentially, rising to 78% of normal 10 days after the initial dose. Thus each successive batch of guinea-pigs was injected with a constant dose (0.03 mg./kg.) of sarin at a mean ChE level higher than that of the preceding batch.

The mortality produced by this dose fell with the rising ChE activity and from Table I it may be deduced that 0.03 mg./kg. sarin would have killed only half of the guinea-pigs between the first and second day after the initial dose, when the mean ChE level was about 40% of its normal value.

TABLE II

THE MEAN % REDUCTION IN BLOOD CHOLINESTERASE ACTIVITY IN GUINEA-PIGS PRODUCED BY A DOSE OF SARIN

The dose of sarin was 0.03 mg./kg. The units of ChE are expressed as $\mu l.CO_2/30$ min./0.1 ml. of whole blood. Substrate, 0.006 M acetylcholine. Method, Warburg manometric. These results were obtained from Experiment 1, Table I.

Initial ChE (Units)	Mean % Reduction	S.E. of Mean	No. of Observations
20– 39	77	3·5	11
40– 59	71	3·1	15
60– 79	69	2·9	16
80– 99	77	1·1	32
100–119	78	1·2	10
120–139	80	2·7	6
140–159	76	2·9	3
Mean % reduction	74·9		

Another point which emerged was that the % reduction of ChE activity was practically independent of the initial ChE level. If the ChE levels, taken immediately before the second injection, are grouped, the % fall produced by 0.03 mg./kg. sarin appears reasonably constant (approximately 75%) though fairly large individual variations occur. This is shown for the first experiment in Table II.

Rabbits

In the foregoing experiments on guinea-pigs, the dose of sarin was kept constant and its effects at various levels of blood ChE were studied and these levels were inseparably associated with varying times after the injection of sarin. A different approach was adopted in the experiments with rabbits in that different doses of sarin were given to rabbits with a lowered blood ChE activity produced by daily injections of one-quarter of the LD50 of sarin.

In a subsequent experiment ethyl pyrophospate was used instead of sarin.

Sarin.—Sixty rabbits were randomized in six groups of 10. Blood, taken from the marginal ear vein of each rabbit, was pooled for each of the six groups. Each ChE determination thus provided an estimate of the mean level of activity for each group of 10 rabbits. Three of the groups then received daily intravenous injections of 0.005 mg./kg. sarin given as 1/10,000 dilution in saline. These repeated sublethal doses of sarin produced marked ChE inhibition without the accompaniment of serious signs or symptoms of sarin poisoning.

The remaining three groups were used as controls and received equivalent injections of saline (0.05 ml./kg.) daily.

One rabbit in each of the sarin treated groups died during the course of the injections; one after the fourth and two after the fifth. One rabbit in the saline series was in a very weak condition and was excluded from the toxicity determinations. The remaining rabbits were in good condition and showed no signs of sarin poisoning.

On the day following the sixth injection, all the surviving animals were again bled and the six pooled samples of blood used for the determination of ChE activity. On the second day after the sixth injection, the LD50 for sarin was determined for each group. Cholinesterase levels in the poisoned and control groups, both before and after the completion of injections, are shown in Table III for two separate but similar experiments.

TABLE III

WHOLE BLOOD CHOLINESTERASE LEVELS IN RABBITS BEFORE AND AFTER SIX INJECTIONS OF SALINE OR OF SARIN

ChE activity, determined by the Michel electrometric method on pooled blood from 10 rabbits in each group, is expressed as ΔpH-90 min./0·5 ml. whole blood × 100. The dose of sarin used was 0·005 mg./kg. Substrate, 0·015 M acetylcholine. For a further explanation of the results see text.

Group	Treatment	Whole Blood ChE Activity				% Inhibition	
		Expt. 1		Expt. 2			
		Before	After	Before	After	Expt. 1	Expt. 2
A	Sarin	169	53	131	61	69	53
B	,,	144	49	139	75	66	46
C	,,	139	54	140	63	61	55
D	Saline	143	120	143	117	16	18
E	,,	150	127	138	112	15	19
F	,,	151	131	137	115	13	16

The degree of inhibition in each poisoned group agreed closely, the mean inhibitions being 66% and 51%. In each experiment there was a small but definite reduction in the saline treated groups, the cause of which is unknown. The values given below for the net inhibition due to sarin have therefore been calculated from the expression $100 (I_p - I_s)/(100 - I_s)$, where I_p and I_s are the observed % inhibitions due to anticholinesterase and saline respectively.

A six point assay of the intravenous toxicity of sarin in the poisoned and control groups was carried out. Fig. 1 shows the dosage/mortality regression lines on log probability co-ordinates for the two experiments. These lines were calculated by Probit analysis (Finney, 1952) using several cycles of iteration. In each case there was no significant departure from parallelism between the control and poisoned groups.

In the first experiment, the values for the intravenous LD50 of sarin for the treated and control groups were 0.013 mg./kg. and 0.020 mg./kg. respectively. The relative toxicity of sarin in the

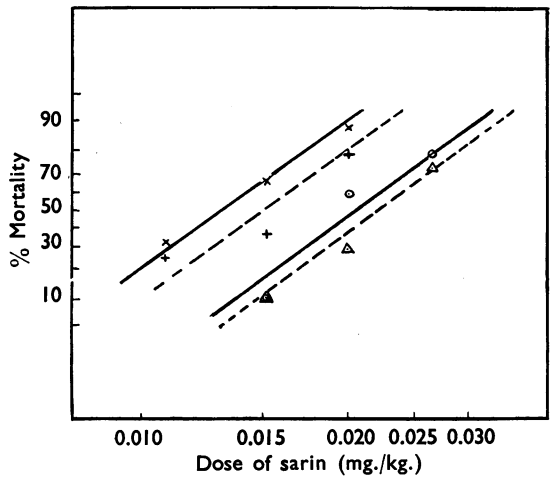

FIG. 1.—Dosage/mortality regression lines for saline and sarin treated rabbits. Expt. 1, X———X sarin treated group; ⊙———⊙ control saline group. Expt. 2, +– – –+ sarin treated group; △– – –△ control saline group.

two groups was thus 1.55 (1.22 to 2.11, $P=0.95$) for a net % ChE inhibition of 60%. The corresponding values for the second experiment were: LD50, treated 0.016 mg./kg., control 0.023 mg./kg., relative toxicity 1.45 (1.09 to 2.07) and net % ChE inhibition 41%. Thus the susceptibility of rabbits to sarin is increased by about 50% when their blood ChE activity is reduced to a half.

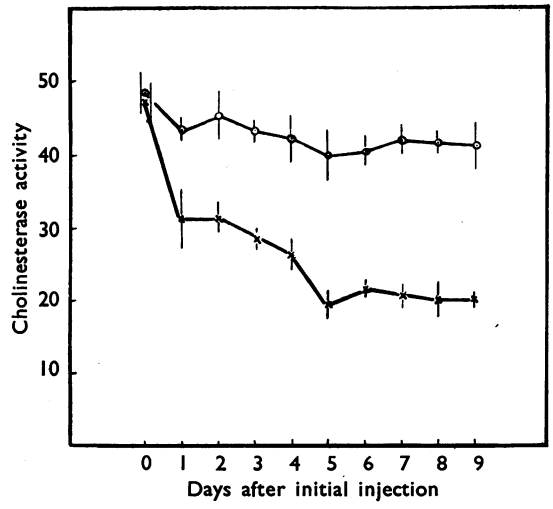

FIG. 2.—Variation in whole blood ChE activity in rabbits receiving daily injections of ethyl pyrophosphate (0.05 mg./kg.) or saline (0.5 mg./kg.). Each point represents the mean value of 6 groups in which blood from 5 rabbits was pooled. The vertical lines indicate the standard deviation. ⊙———⊙, saline group; X———X, ethyl pyrophosphate group.

Ethyl Pyrophosphate.—In these experiments ethyl pyrophosphate (TEPP) was used instead of sarin. Sixty rabbits were randomized, this time in twelve groups of 5, and venous blood from each rabbit was pooled for each of the 12 groups. Six of the groups received daily intravenous injections of 0.05 mg./kg. of TEPP in saline (0.1 mg./ml.). The remaining six groups received daily injections of saline (0.5 ml./kg.).

One rabbit in the TEPP treated group died during the conditioning period and reference to it was excluded from the records. The remaining animals showed no cholinergic symptoms and were in good condition.

The injections of TEPP produced a progressive fall in blood ChE activity up to the fifth day, after which it remained steady at about 40% (Fig. 2).

On the day following the ninth injection final determinations of the ChE activity were made and the LD50 for TEPP was determined for each of the two groups. At this stage the ChE inhibition of the TEPP injected animals was 57.5%. Saline injection again produced a reduction in ChE activity (13.9%) which was comparable with that found in the sarin experiments (Table IV).

TABLE IV

WHOLE BLOOD CHOLINESTERASE LEVELS IN RABBITS BEFORE AND AFTER SIX INJECTIONS OF SALINE OR ETHYL PYROPHOSPHATE (0.05 MG./KG.), AND INTRAVENOUS LD50 OF ETHYL PYROSPHOSPHATE FOLLOWING THE FINAL INJECTION

The units of ChE activity, expressed as the mean ±S.D. of 6 groups, each comprising pooled blood from 5 rabbits, are ΔpH/hr. × 100, determined by the Michel method using 0·2 ml. blood with 0·015 M ACh as substrate.

Injection of	Whole Blood ChE Activity		% Inhibition	i.v. LD50 Ethyl Pyrophosphate (mg./kg.)
	Before	After 9th Injection		
Ethyl pyrophosphate	47·0±2·5	20·0±0·9	57·5	0·305 (0·243 to 0·382)
Saline ..	48·2±2·7	41·5±3·3	13·9	0·428 (0·400 to 0·456)

Fig. 3 shows the dosage/mortality regression lines on log probability co-ordinates for the final TEPP assay. As for sarin, these lines were calculated by probit analysis using several cycles of iteration. Unlike the sarin regression lines, however, those for TEPP were significantly non-parallel. As a result of this lack of parallelism independent regression lines were calculated and the toxicity ratio between control and poisoned groups obtained for the LD50 only. This toxicity ratio, LD50 Control Group/LD50 TEPP Group, was 1.40 (1.11 to 1.77).

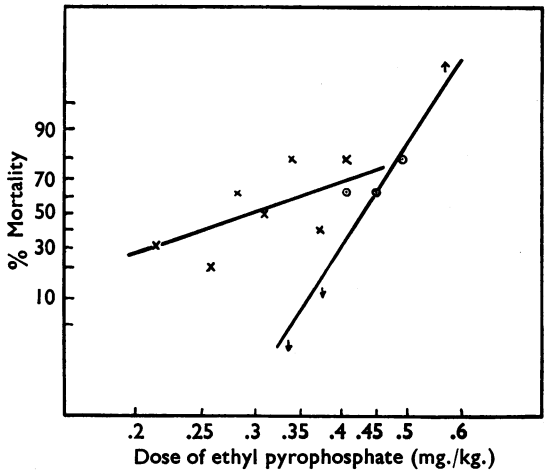

FIG. 3.—Dosage/mortality regression lines for saline and ethyl pyrophosphate treated rabbits X———X ethyl pyrophosphate treated group. ⊙———⊙ controls saline treated.

DISCUSSION

The experiments described above show that the toxicity of sarin or TEPP to the guinea-pig and the rabbit is increased when the ChE activity of the whole blood is markedly reduced below normal by prior treatment with the respective agents. These results would imply therefore that an increased susceptibility to an anti-ChE may be developed by previous poisoning with doses which have shown no clinical signs or symptoms and that the level of the blood ChE is an index of this susceptibility.

In guinea-pigs, the blood ChE was reduced by a single sublethal dose of sarin, and the regeneration of ChE activity was used to provide different enzyme levels. It was found that when the blood ChE returned to about 40% of normal, the same sublethal dose of sarin (equivalent to about 70% of the normal LD50) had become the LD50 dose.

In the rabbit experiments where the ChE activity was reduced by daily injections of sarin or TEPP, a similar result was obtained. Thus, in the first sarin experiment, when the ChE was reduced to 40% the LD50 was reduced to 65% of normal; in the second, a reduction of ChE to 59% was accompanied by a fall in the LD50 to 70%. TEPP produced a reduction of 50% in ChE corresponding with an LD50 which was 72% of normal.

Thus, the approximate quantitative relationship throughout appears to be that susceptibility is increased in the ratio 1.5 when the blood ChE activity is reduced to half. All these results agree very closely when considered in relation to the reduction in LD50 which may be associated with a given reduction in blood ChE activity. However, they have somewhat fortuitously centred around a fixed value for the ChE fall. If, however, the guinea-pig experiments are considered, it may be seen from Table I that there is, in fact, a very close correlation between mortality and ChE level over the range 20% to 80% of normal.

It should be emphasized that the level of blood ChE is no indication of the level in other tissues, and is only an empirical indication of a general state of increased sensitivity of the whole animal towards anti-ChE. In all the experiments described, ChE levels were determined upon whole blood, namely upon a mixture of the true- and pseudo-ChE, and it is by the use of whole blood analyses that the association between reduced ChE activity and increased susceptibility has been established. This is a matter of some practical importance, since it is much more convenient to determine ChE activity in whole blood than in separated cells or plasma.

One final point remains to be discussed, that is the difference between the slopes of the regression lines in the TEPP experiment compared with the marked parallelism in the sarin experiment. Differences in slope between dosage mortality regression lines frequently reflect differences in the mode of action of the toxic agents. In our experiments it would appear that the toxic effects of sarin are the same, whether the animal has been pre-treated with sublethal doses of sarin or not. Pre-treatment with TEPP, however, may have modified the mode of toxic action of subsequent doses of TEPP in such a way as significantly to have changed the slope. This difference might be related to differences in stability between the phosphorylated enzymes (Hobbiger, 1956).

The quantitative aspects of the similarity between sarin and TEPP in the increased susceptibility following pre-treatment have been derived from comparison at the LD50. If, however, the differences in slope are taken into account, as may be seen from Fig. 3, animals with lowered blood ChE activity following daily TEPP injections may be even more susceptible to low doses of this agent, namely half the normal LD50 (0.2 mg./kg.), than are the corresponding sarin treated animals, while at doses above the LD50 there may be a corresponding relative increase in the comparative survival rate. Finally, although there are differences in detail between the TEPP and sarin effects, the general conclusion remains, that an increase

in sensitivity to poisoning by either of these agents of the normal LD50) had become the LD50 dose. occurs when they are used to lower the blood ChE activity by repeated injections of sublethal quantities. In these experiments, the lowering of blood ChE was produced by the same agent as that with which the toxicity assessment was carried out. A problem requiring further investigation is whether a similar relationship exists, when the lowering of enzyme is produced by one agent and the final poisoning by another.

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