Biochemical Pharmacology, Vol. 18, pp. 813–820. Pergamon Press. 1969. Printed in Great Britain

THE INFLUENCE OF TRIORTHOCRESYLPHOSPHATE ON THE DISTRIBUTION OF ^{32}P IN THE BODY OF THE RAT AFTER THE INJECTION OF ^{32}P-SARIN

R. L. POLAK and E. M. COHEN

Medical Biological Laboratory of the National Defence Research Organization TNO, Lange Kleiweg 139, Rijswijk Z.H., The Netherlands

Abstract—The ChE- and AE- activities and the ^{32}P-concentration of the plasma from rats 1 and 24 hr after s.c. injection of ^{32}P-sarin (200 μg/kg) were measured. One hr after the injection the ChE was inhibited to approx. 10 per cent and the AE to 40–50 per cent; 23 hr later 50 per cent of the ChE activity was found to be restored and the AE had recovered its full activity. One hr after the ^{32}P-sarin injection about 12 per cent of the injected amount of ^{32}P was circulating in the blood plasma, but 23 hr later the radioactivity had disappeared nearly completely from the plasma.

Pretreatment of rats with TOCP, an irreversible inhibitor of the plasma AE, enhanced the toxicity of sarin 6–8 times. It also produced a shift of the radioactivity found in the body of the rat 1 hr after the injection of ^{32}P-sarin from the plasma to the brain, the muscles, the lungs and the kidneys.

Serum from normal and from TOCP treated rats was incubated with ^{32}P-sarin in different concentrations and thereafter filtered through Sephadex columns in order to determine the amounts of ^{32}P-sarin attached to the proteins in the serum. It appeared that the proteins in the serum from TOCP treated rats had lost a considerable part of their ^{32}P-sarin binding capacity.

Electrophoresis of serum from a rat injected with ^{32}P-sarin on cellulose-acetate and subsequent autoradiography of the cellulose-acetate strip showed radioactivity localised on a spot with AE- activity.

It was concluded that the plasma proteins with which the ^{32}P-sarin combined were identical with AE. The sarin binding capacity of the plasma AE was, however, considered to be too small to fully account for the enhanced toxicity of sarin for rats treated with TOCP. Non-specific sarin binding sites with affinity for TOCP elsewhere in the body were postulated.

IN A PREVIOUS short communication on the distribution of ^{32}P in the body of the rat after injection of ^{32}P labelled organophosphates[1] it was demonstrated that after the administration of a sublethal dose of ^{32}P-sarin or ^{32}P-soman to the atropinized rat part of the injected radioactive material became attached to plasma proteins from which it could be released by DAM* but not by P-2-AM. Earlier Myers[2] had found that DAM is a much better reactivator of sarin inhibited rat plasma AE than P-2-AM. In contrast to P-2-AM, DAM is not very effective in reactivating sarin inhibited ChE.[3] Therefore it seemed likely that the plasma proteins with which the ^{32}P labelled nerve gases were found to combine might be identical with AE. This was already suggested by Myer's observation, that rats in which the plasma AE had been blocked

* The following abbreviations will be used throughout this paper: ChE (cholinesterase), AE (aliesterase = tributyrinase), sarin (isopropyl methylphosphonofluoridate), soman (pinacolyl methylphosphonofluoridate), P-2-AM (pyridine-2-aldoxime methiodide), MINA (monoisonitrosoacetone), DAM (diacetylmonoxime), TOCP (triorthocresylphosphate).

irreversibly by pretreatment with a non-lethal dose of TOCP, had become much more sensitive to the lethal action of sarin than normal animals.

It seemed of interest to study the influence of TOCP on the distribution of radioactive material in the body of the rat after injection of ^{32}P-sarin and on the sarin binding capacity of the plasma proteins in order to relate our previous results to those described by Myers.

METHODS

Materials

The ^{32}P-sarin was synthetized by the Chemistry Section of Defence Research Establishment, Suffield, Ralston, Alberta, Canada*. The specific activity at the time of arrival at Rijswijk varied between 50 and 120 ml/g. Immediately after arrival a stock solution of the nerve gas in a concentration of approximately 10^{-2}M in anhydrous isopropyl alcohol was prepared. This stock solution was stored at $-20°$. The purity of the compound, determined a few days after arrival in this laboratory, varied between 90 and 97 per cent. Atropine sulphate, acetylcholine chloride, glycerol tributyrate and a crude preparation of TOCP were commercially obtained. Male and female albino rats weighing 180–250 g from one of the strains of this laboratory were used.

The distribution of ^{32}P in the body of the rat

The ^{32}P-sarin was dissolved in a 0·9% NaCl solution and injected in a volume of 2·5 ml/kg either subcutaneously or in an exposed saphenous vein under ether anaesthesia. In all animals an aqueous solution of atropine sulphate (36 mg/kg) was administered intraperitoneally 1·5 min after the nerve gas.

One hr or 24 hr after the ^{32}P-sarin injection the animals were killed by bleeding from a carotid artery. This haemorrhage was preceded by an i.v. injection under ether anaesthesia of 0·1 ml of a 5 per cent heparine solution. The blood was collected in graduated test tubes and centrifuged at 3000 r.p.m., during 10 min in order to separate the red cells from the plasma. The erythrocytes were not rinsed. The other organs were weighed and wet-ashed with equal parts of concentrated nitric acid and sulphuric acid and the clear fluids thus obtained were made up to 10 or 20 ml with demineralized water. The radioactivity of the blood and the wet-ashed organs was measured in a Geiger–Müller liquid counter and compared on the same day with that of a standard solution prepared from the used ^{32}P-compound.

The measured amounts of ^{32}P were expressed as mμg ^{32}P-sarin per gram wet organ weight or per ml blood.

The statistical significance at the 5 per cent level of the differences between the ^{32}P-contents of the organs of TOCP pretreated and untreated animals injected with ^{32}P-sarin was determined by the rank sum test.[4]

Experiments with Sephadex columns

From solutions of different concentrations of ^{32}P-sarin in saline 0·1 ml was added to each of four 0·9 ml samples of serum from one rat and the mixtures were incubated during 30 min at 37°. Thereafter 0·9 ml of each mixture was fractionated through a

* Thanks are due to the Defence Research Board of Canada for providing us with this material.

Sephadex G-50 column (height 30 cm, dia. 2 cm), saturated with 0·01 M Tris-buffer (pH = 7·8) in saline. Tris-buffer in saline was also used for elution. The amounts of ^{32}P in the micro- and macro-molecular fractions were expressed as percentages of the total amounts of ^{32}P applied to the columns. These experiments were performed with serum from normal rats and with serum from rats which 24 hr earlier had received a s.c. injection of TOCP (40 or 80 mg in 0·5 or 1 ml arachid oil per kg respectively).

The toxicity of sarin in TOCP pretreated animals

In determinations of the toxicity of sarin for rats which 24 hr earlier had received an i.p. injection of 1 ml/kg of a solution of 80 mg TOCP per ml arachid oil, the percentages of animals killed at different dose levels were plotted on log-probit paper and the LD_{50} was estimated by the method described by Litchfield and Wilcoxon.[5] At each dose level 8 rats were used. In contrast to the other experiments with sarin the animals used in these toxicity trials did not receive atropine.

Determinations of the ChE- and AE-activities

The ChE- and AE-activities of plasma and the ChE-activity of brain tissue from rats treated with TOCP or with sarin were determined by the Warburg method at 37° in a Krebs–Ringer-bicarbonate medium, equilibrated with a mixture of 95 per cent N_2 and 5 per cent CO_2 (pH = 7·4). Atropine sulphate (36 mg/kg) was administered 1·5 min after the sarin injection. For the ChE determinations 0·75 ml of a 10 per cent brain homogenate or 0·75 ml undiluted plasma and for the AE determinations 0·75 ml 1:5 diluted plasma was pipetted into the main compartment of the manometric vessel and made up to 2·75 ml with Krebs–Ringer-bicarbonate solution. The side arm contained 0·25 ml of one of the following solutions: 200 mg ACh in 9 ml Krebs in the ChE determinations and 0·48 ml glycerol tributyrate plus 240 mg gum arabic in 10 ml Krebs in the AE determinations. The final concentration of ACh was 10^{-2} M, that of glycerol tributyrate $1{\cdot}3 \times 10^{-2}$ M. Presented are means ± S.E.M. of 4 or 6 observations based on duplicate determinations of enzyme activity. The statistical significance of differences at the 5 per cent level was determined using Welch's *t*-test.[6]

Electrophoresis

Electrophoresis of serum from a rat, which had been injected i.v. with 50 μg/kg of ^{32}P-sarin 10 min before bleeding, was performed on cellulose-acetate strips. After fixation and staining the strips were autoradiographed. Kodak Medical X-ray Film, blue brand, was used.

RESULTS

The AE- and ChE-activities of the blood plasma 1 and 24 hr after injection of ^{32}P-sarin

One hr after the s.c. injection of 200 μg sarin/kg into rats the plasma AE-activity amounted to approx. 35 per cent and the plasma ChE-activity to about 20 per cent of that of the control animals. Twenty-three hr later the AE-activity was no longer significantly inhibited and the ChE-activity had risen to about 50 per cent (Table 1). The inhibition of AE 1 hr after the ^{32}P-sarin injection, the inhibition of ChE 1 and 24

hr after the administration of the organophosphorus compound and the spontaneous reactivation of the enzymes which had taken place in 24 hr, were all statistically significant.

TABLE 1. THE ChE- AND AE-ACTIVITIES OF PLASMA FROM RATS 1 AND 24 hr AFTER S.C. INJECTION OF 200μg SARIN/kg

		Control (A)	Sarin (B)	% activity (C)
ChE	1 hr	1027 ± 44 (12)	191 ± 22 (11)	19
	24 hr	1060 ± 33 (12)	496 ± 14 (10)	47
AE	1 hr	5741 ± 338 (12)	1954 ± 177 (12)	34
	24 hr	5960 ± 247 (12)	5410 ± 186 (10)	91

In the columns (A) and (B) the enzyme activities are expressed in terms of CO_2 production (μl/ml/hr). The figures represent means of 10–12 determinations in duplicate ± S.E.M. The values in the column (C) are obtained by the equation (B)/(A) × 100.

The ^{32}P-concentration of the blood plasma 1 and 24 hr after the injection of ^{32}P-sarin

One hr after the s.c. injection of 200 μg of ^{32}P-sarin/kg into rats the ^{32}P-concentration of the blood plasma averaged the equivalent of 372 mμg ^{32}P-sarin/ml blood; 23 hr later however the plasma contained only 4 mμg/ml blood (see Table 2). This nearly

TABLE 2. THE ^{32}P-ACTIVITIES OF THE PLASMA AND THE RED CELLS FROM RATS 1 AND 24 hr AFTER S.C. INJECTION OF 200 μg ^{32}P-SARIN/kg

	Plasma	Erythrocytes	Total blood
1 hr	372 ± 12	123 ± 3·5	495
24 hr	3·6 ± 2·4	49 ± 2·0	53

The activities are expressed as m μg ^{32}P-sarin per ml blood. Means of 9 observations ± S.E.M. The values for the total blood were obtained by adding the plasma and red cell values.

total disappearance of the plasma radioactivity parallels the nearly complete spontaneous reactivation of the AE which had taken place 24 hr after the injection (see Table 1), but not the only very incomplete recovery of the ChE-activity at that time. These results therefore do not conflict with the assumption that the bulk of the ^{32}P present in the plasma 1 hr after the injection of ^{32}P-sarin into the rat is attached to plasma AE.

To test this idea further experiments were performed on rats in which the plasma AE was inactivated irreversibly by pretreatment with TOCP.

Influence of TOCP on brain ChE- and plasma ChE- and AE-activities

As shown in Table 3 TOCP in doses of 40 and 80 mg/kg respectively did not significantly influence the ChE-activity of the brain and of the blood plasma, but produced a substantial inactivation of the plasma AE. This confirms earlier findings.[2]

Enhancement of the toxicity of sarin for rats by TOCP

In agreement with the findings of Myers[2] TOCP in doses in which it produced no apparent ill effects strongly enhanced the toxicity of sarin for rats. In rats treated 24 hr

earlier with a s.c. injection of 40 or 80 mg TOCP/kg the LD_{50} of s.c. injected sarin was 41 (36–46)* μg/kg and 25 (21–29) μg/kg respectively, whereas it amounted to 210 (184–239) μg/kg in untreated rats. The dose–response curves of the sarin effect in untreated and pretreated rats were parallel.

TABLE 3. THE INFLUENCE OF TOCP ON THE CHE-ACTIVITY OF THE BRAIN AND THE CHE- AND AE-ACTIVITIES OF THE BLOOD PLASMA

Enzyme	Dose TOCP (mg/kg)	Dose arachid oil (mg/kg)	Number of observations	Arachid oil (control) (A)	Arachid oil + TOCP (B)	% Activity $\frac{(B)}{(A)} \times 100$
Brain ChE	40	0·5	4	1116 ± 11	1152 ± 37	104
	80	1·0	6	1090 ± 28	1128 ± 30	101
Plasma ChE	40	0·5	9	1083 ± 43	1027 ± 59	95
	80	1·0	12	1058 ± 40	1031 ± 31	97
Plasma AE	40	0·5	9	4647 ± 429	1820 ± 204	39
	80	1·0	12	4996 ± 308	618 ± 89	12

Enzyme activities of brain and plasma from animals, 24 hr after s.c. injection of either arachid oil only (A) or of arachid oil with TOCP (B), expressed as μl CO_2 produced per gram brain tissue or per ml plasma during 1 hr. Means ± S.E.M.

Influence of pretreatment with TOCP on the distribution of ^{32}P in the body of the rat after injection of ^{32}P-sarin

One hr after the s.c. injection of 25 μg ^{32}P-sarin/kg into rats, which 24 hr earlier had received a s.c. injection of 40 or 80 mg TOCP/kg, much less ^{32}P was found in the blood plasma and significantly more in the brain, the lungs, the kidneys and the m.gastrocnemius than in control animals, pretreated with arachid oil without TOCP. Both groups of animals had been injected with 36 mg/kg of atropine sulphate 1·5 min after the sarin. The amounts of ^{32}P in the liver and erythrocytes of TOCP pretreated animals were not significantly different from those in control animals. These results are illustrated in Table 4 which in addition shows that 24 hr after the ^{32}P-sarin injection the radioactivity in the liver and in the erythrocytes had decreased to a much smaller extent in the TOCP pretreated animals than in the control animals.

The ^{32}P-sarin binding capacity of serum proteins

A few experiments were performed in which the capacity of the proteins in rat serum to bind ^{32}P-sarin *in vitro* was studied. Various samples of serum from normal rats and from rats pretreated with TOCP were incubated with ^{32}P sarin during 30 min and thereafter fractionated through Sephadex G-50 columns. The percentage of radioactivity eluted together with the large molecules was considered to be a measure of the percentage of the ^{32}P-sarin which had been bound to the serum proteins.

The relative amount of ^{32}P eluted in the macromolecular fractions appeared to be dependent on the quantities of ^{32}P-sarin added to the serum (see Table 5). When samples of normal rat serum had been incubated with ^{32}P-sarin in a final concentration of 0·25 and 1 μg/ml, 42–45 per cent of the radioactivity appeared in the macromolecular fractions. When the serum was incubated with ^{32}P-sarin in higher final

* 95 per cent confidence limits.

concentrations, a smaller percentage of the ^{32}P-sarin was bound to the proteins. This observation suggests that the sarin binding capacity of the protein is limited. It appeared that no more than 18 per cent of 4μg ^{32}P-sarin, that is 0·72 μg ^{32}P-sarin, could be attached to the proteins of 1 ml 90 per cent serum. This would mean that maximally about 0·8 μg ^{32}P-sarin (= 5·7 m μmoles) could be bound to the proteins of 1 ml undiluted serum.

TABLE 4. THE INFLUENCE OF PRETREATMENT WITH TOCP ON THE DISTRIBUTION OF ^{32}P IN THE BODY OF THE RAT 1 AND 24 hr AFTER THE S.C. INJECTION OF 25 μg ^{32}P-SARIN/kg

	1 hr after injection ^{32}P-sarin			24 hr after injection ^{32}P-sarin	
	Control	TOCP (40 mg/kg)	TOCP (80 mg/kg)	Control	TOCP (40 mg/kg)
Blood	107 ± 2 (10)	**43** ± 2 (5)	**35** ± 3 (6)	8 ± 0·9 (4)	**15** ± 1 (5)
Plasma	89 ± 2 (10)	**23** ± 2 (5)	**18** ± 3 (6)	0·9 ± 0·1 (4)	**0·3** ± 0·1 (5)
Red cells	18 ± 1 (10)	20 ± 1 (5)	16 ± 0·4 (6)	7 ± 0·9 (4)	**15** ± 1 (5)
Brain	3 ± 0·2 (10)	**32** ± 2 (5)	**26** ± 2 (6)	2 ± 0·5 (4)	**22** ± 3 (5)
Liver	24 ± 1 (10)	27 ± 2 (5)	28 ± 2 (6)	2 ± 0·5 (4)	**11** ± 2 (5)
Lungs	40 ± 2 (6)		**51** ± 4 (6)		
Kidneys	88 ± 11 (6)		**153** ± 8 (6)		
M. gastrocn.	4 ± 0·3 (6)		**7** ± 0·8 (6)		

The ^{32}P- content of the blood, the plasma and the red cells is expressed as mμg ^{32}P-sarin per ml blood, that of the other organs per g organ wet weight. Presented are means ± S.E.M. followed by the number of observations between brackets. Twenty four hr before the ^{32}P-sarin injection the animals received a s.c. injection of either TOCP in arachid oil (40 ml/kg or 80 mg/ml/kg) or of the same volume of the solvents. Since the results of the control animals injected with 0·5 ml arachid oil/kg were not different from those injected with 1 ml arachid oil/kg, they were pooled. One and a half min after the ^{32}P-sarin 36 mg atropine/kg was injected intraperitoneally. Values significantly different from the control values (rank sum test, Wilcoxon) are in bold type.

TABLE 5. THE INFLUENCE OF TOCP ON THE ^{32}P-SARIN BINDING CAPACITY OF RAT SERUM PROTEINS

Final concentration ^{32}P-sarin (μg/ml)	0·25	0·5	1	2	4
Normal serum	42	44	45	30	
		45	43	31	18
Serum from a TOCP (40 mg/kg) treated rat		10·1	6·6	5·1	4·1
Serum from a TOCP (80 mg/kg) treated rat	1·2	3·3	2·4	2·9	

Each horizontal row of figures gives the results of an experiment in which four portions of serum from one rat had been incubated during 30 min at 37° with ^{32}P-sarin in different concentrations before being fractionated through Sephadex columns. The figures represent the amounts of ^{32}P appearing in the macromolecular fractions, expressed as percentages of the total quantity of ^{32}P-sarin added to each sample of serum. Each figure is the result of one observation. The TOCP was injected s.c. into the rats 24 hr before haemorrhage.

The proteins of TOCP pretreated rats appeared to have lost a considerable part of their ^{32}P-sarin binding capacity. In the 90 per cent serum of a rat, which 24 hr earlier had received a TOCP injection of 40 mg/kg, maximally 4·1 per cent of 4 μg, that is 0·16 μg/ml was eluted with the macromolecular fraction. This would be equivalent to

0·18 μg/ml undiluted serum. The serum proteins from a rat treated with 80 mg/kg of TOCP appeared to have lost a still more important part of their ability to bind sarin. The table shows that the absolute amounts of ^{32}P bound by plasma proteins from TOCP treated rats increase with increasing ^{32}P-sarin concentrations. This suggests that other plasma proteins than AE are not entirely devoid of sarin binding activity.

Electrophoresis of serum from a rat after injection of ^{32}P-sarin

Electrophoresis on cellulose-acetate strips of serum, taken from a rat which was bled 10 min after the i.v. injection of 50 μg/kg of ^{32}P-sarin, was carried out and after fixation and staining the cellulose-acetate strip was autoradiographed. Radioactivity was found in the α-globulin fraction only.

DISCUSSION

One hr after the injection of 200 μg/kg of ^{32}P-sarin into the rat both the AE-activity and the ChE-activity of the plasma were definitely inhibited; 23 hr later the AE-activity had recovered to a large extent whereas the ChE-activity was still inhibited by about 50 per cent. At the same time nearly all ^{32}P had disappeared from the plasma. Apparently the amount of ^{32}P-sarin involved in this substantial inactivation of ChE were so small that they could not be measured with the method used.

The idea that a considerable part of the ^{32}P in the plasma was attached to AE is supported by different findings:

(1) there appears to be a parallel between the nearly complete reactivation of the AE and the nearly total disappearance of the ^{32}P from the plasma 24 hr after the injection of ^{32}P-sarin;

(2) plasma from rats pretreated with TOCP in a dose, which caused a nearly complete and probably selective inactivation of plasma AE, had lost most of its capacity to bind sarin;

(3) radioactivity was shown to be localized exclusively in the α-globulin fraction in an electrophoretogramme of serum from a ^{32}P-sarin treated rat; this fraction has been shown to have AE-activity.[7] It must be stressed, however, that the autoradiographic technique used yielded qualitative information only, and that in the experiments in which serum from TOCP pretreated rats was incubated with ^{32}P-sarin the relatively small amounts of ^{32}P bound to serum proteins increased with increasing ^{32}P-sarin concentrations. The latter observation suggests that other plasma proteins than AE also may be involved, especially with higher doses of sarin.

The Sephadex experiments provided some data about the sarin binding capacity of rat plasma proteins. Maximally 0·8 μg ^{32}P-sarin or 5·7 mμmoles could be attached to the proteins in 1 ml serum, that is about 0·4 μg or 2·8 mμmoles/ml blood. This would amount to 65 × 0·4 μg = 26 μg sarin combining with plasma proteins per kg rat, since the total blood volume is 6·5 per cent of the body weight.[8]

The finding that the radioactivity in the brain from TOCP treated rats after the injection of ^{32}P-sarin was much higher than that in the same organ from animals not treated with TOCP, supports Myer's idea that TOCP enhances the toxicity of sarin for the rat by occupying certain non-vital sarin binding sites, thus allowing a larger part of the injected nerve gas to penetrate into the more vital centres. However, should the plasma proteins of the rat have a sarin binding capacity of 26 μg/kg, as

calculated above, the reduction by TOCP of the LD_{50} of sarin from 210 μg/kg to 25 μg/kg cannot be explained by TOCP having occupied the plasma proteins only as proposed by Myers. It would appear that sarin binding non-ChE sites elsewhere in the body were occupied by TOCP as well.

It is not clear why 1 hr after the injection of ^{32}P-sarin the radioactivity in the livers and red cells of TOCP animals was not different from that in animals to which no TOCP had been administered whereas it was much higher 24 hr after the injection.

Acknowledgements—The authors gratefully acknowledge the skilful technical assistance of Mrs. Maria M. Bertels-Meeuws and Miss Lisa Mobach.

REFERENCES

1. R. L. POLAK, *Acta Physiol. Pharmacol. Neerl.*, **12**, 81 (1963).
2. D. K. MYERS, *Biochim. biophys. Acta* **34**, 555 (1959).
3. B. M. ASKEW, *Br. J. Pharmac. Chemother.* **11**, 417 (1956).
4. F. WILCOXON, *Biometr. Bull.* **1**, 80 (1945).
5. J. F. LITCHFIELD and F. WILCOXON, *J. Pharmac. exp. Ther.* **96**, 99 (1949).
6. B. L. WELCH, *Biometrika*, **34**, 18 (1947).
7. P. J. CHRISTEN, personal communication.
8. E. J. FARRIS and J. Q. GRIFFITH, *The Rat in Laboratory Investigation*, p. 413. Lippincott, Philadelphia (1949).