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THE BINDING OF SARIN IN THE BLOOD PLASMA OF THE RAT

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Abstract—Sephadex filtration of serum from rats 10 min after injection of 50 μg/kg of ^{32}P-sarin demonstrated that about 70 per cent of the radioactivity present in the serum was attached to molecules with a molecular weight above 10,000. Incubation of samples of this serum with oximes caused a decrease in the amounts of ^{32}P attached to the large molecules and an increase in those appearing in the micromolecular fractions. The oximes apparently released ^{32}P from the large molecules. DAM and MINA were much more effective in this respect than P-2-AM and TMB-4.

The activities of the ChE and AE in serum from rats 10 min after injection of sarin were determined. Both enzymes were partly inactivated. The AE reactivating potencies of the oximes concurred with their ^{32}P releasing effects: DAM and MINA (0·5 mM) partly reactivated the AE, but P-2-AM and TMB-4 did not. In contrast TMB-4, P-2-AM and MINA produced a partial reactivation of the ChE whereas DAM had no effect.

It was concluded that the obtained results support the idea that the large molecules in the serum to which the radioactivity was attached were identical with AE.

IN THE preceding[1] paper the distribution of ^{32}P in the body of the rat 1 hr after the i.v. injection of ^{32}P-sarin* and the way this distribution is influenced by the i.p. injection of oximes were studied. It was observed that about 18 per cent of the injected dose of nerve gas was present in the circulating blood plasma. The amounts of radioactive material in the plasma decreased immediately after the injection of DAM or MINA, but not after that of P-2-AM. These observations were explained on the basis of our earlier findings[2] that the radioactive materials in the plasma of rats after injection of ^{32}P-sarin are kept within the vascular space by being attached to the plasma AE. It was demonstrated that DAM and MINA (in contrast to P-2-AM) reactivate this enzyme. Part of the liberated and hydrolyzed[3] ^{32}P-sarin was assumed to leave the vascular space by diffusion. If such a process takes place *in vivo*, it should be possible to reproduce it *in vitro*. This was attempted in the present experiments in which serum from rats after injection of ^{32}P-sarin was incubated during 15 min with either saline or one of the oximes DAM, MINA, P-2-AM and TMB-4 and subsequently subjected to Sephadex filtration. In parallel experiments the ChE- and AE-activities of serum from sarin-treated rats were determined after pre-incubation with either saline or one of these oximes.

MATERIALS

The same materials were used as in the preceding paper. Furthermore TMB-4

* The same abbreviations were used as in the preceding paper.

(1,1'-trimethylenebis(4-hydroxyiminomethylpyridinium)dibromide) was used. It was synthetized by the Chemical Laboratory RVO-TNO, Rijswijk (ZH), The Netherlands.

METHODS

Experiments with Sephadex columns. Female albino rats (198–210 g) received an i.v. injection of 50μg/kg of ^{32}p-sarin and 1·5 min later an i.p. injection of 36 mg/kg atropine. The animals were killed by bleeding 10 min after the injection of the nerve gas. In each experiment five 1 ml samples were taken from a well-mixed pool of serum from two rats. To each 1 ml sample either 0·5 ml of a solution of 0·9 per cent NaCl in 0·01 M Tris-buffer (pH 7·8) or 0·5 ml of a solution of DAM, MINA, P-2-AM or TMB-4 in 0·01 M Tris-buffer was added and the mixtures were incubated during 15 min at 37°. Thereafter 1 ml of each incubation mixture was applied to a Sephadex G50 column. The columns were 30 cm high and had a diameter of 2 cm. They were saturated with 0·01 M Tris-buffer in saline (pH = 7·8) which was also used for elution. Approximately 180 ml was needed to elute all radioactivity. Fractions with a molecular weight of less and more than 10,000 were thus separated. The amounts of radioactivity found in the micro-molecular fractions were expressed as percentages of the total amounts of ^{32}P present in the samples of serum applied to the columns. Serum was used instead of plasma after it had been found in pilot experiments that plasma and serum gave the same results.

Enzyme activities. Female albino rats (166–204 g) received an i.v. injection of 50 μg/kg of sarin and 1·5 min later an i.p. injection of 36 mg/kg of atropine. The animals were killed by bleeding 10 min after the injection of the nerve gas. In each experiment the serum from five sarin-injected rats was pooled. In order to measure the ChE- and AE-activities of the serum after an incubation period comparable to that in the Sephadex experiments, the conventional Warburg method was slightly modified: the substrate solution (0·25 ml of 200 mg ACh in 9 ml Krebs–Ringer solution for the ChE-determination and 0·25 ml of 0·48 ml glycerol tributyrate plus 240 mg gum arabic in 10 ml Krebs–Ringer solution for the AE-determination) was pipetted into the main compartment of the Warburg vessel and the necessary amount of Krebs–Ringer solution (final volume after mixing of the substrate and enzyme solutions 3 ml) was added to this compartment. Next samples of the pooled serum were mixed with either 0·9 per cent NaCl or one of the oximes in 0·01 M Tris-buffer in a volume to volume ratio of 2:1. The final oxime concentration was 0·5 mM. Simultaneously a sample of serum from an untreated rat was mixed with 0·9 per cent NaCl in the same volume to volume ratio as the other samples. From the incubation mixtures 0·75 ml or 0·25 ml was pipetted into the side arms of the Warburg vessels for the ChE- and AE-determinations, respectively. Thereafter the vessels were attached to the manometers and heated to 37°. Subsequently a stream of 95 per cent N_2 and 5 per cent CO_2 was conducted through the vessels under continuous shaking. Next the manometer-vessel systems were closed, the substrate and enzyme solutions were mixed and the measurement of the enzyme activities was started. As a result of the time taken by the different manipulations the enzymes were incubated during about 7 min at room temperature and during about 11 min at 37° before the determination of the enzyme activities started.

Statistical evaluation of the results. The statistical significance of differences between mean enzyme activities was determined by Welch's *t*-test.[4]

RESULTS

Sephadex filtration. When serum from rats injected with ^{32}P-sarin was applied to Sephadex columns after 15 min incubation with 0·9 per cent NaCl, approximately 70 per cent of its radioactivity was eluted in the macromolecular fractions, which means that this percentage of the ^{32}P in the serum was attached to large molecules (see Table 1). After incubation with one of the oximes higher percentages appeared in the micromolecular fractions and lower percentages in the macromolecular fractions, indicating that the oximes released ^{32}P from the large molecules. The magnitude of this

TABLE 1. THE PERCENTAGES OF THE RADIOACTIVITY PRESENT IN SERUM FROM ^{32}P-SARIN-TREATED RATS, WHICH APPEARED IN THE MACROMOLECULAR FRACTIONS WHEN THIS SERUM WAS FILTRATED THROUGH SEPHADEX G 50 COLUMNS AFTER INCUBATION WITH A SOLUTION OF EITHER 0·9% NaCl (control) OR ONE OF THE OXIMES TMB-4, P-2-AM, MINA AND DAM IN TRIS-BUFFER

Final conc. oxime (mM)	Results					^{32}P conc. serum (mμg/ml of ^{32}P-sarin)
	Control	TMB-4	P-2-AM	MINA	DAM	
2	76	55	48	9	3	420
1	69	56	53	20	12	432
0·5	69	60	57	22	9	422
0·25	66	63	62	44	36	411
0·125	70	66	67	51	41	496

Each figure represents one observation and each horizontal series of figures is the result of one experiment with five samples of serum taken from a well-mixed pool obtained from two rats. Since there was a complete recovery of the applied amounts of radioactivity, the percentages appearing in the micromolecular fractions can be calculated by subtracting the presented values from 100.

effect was dependent on the concentration and on the nature of the oxime. In concentrations between 0·5 and 0·125 mM TMB-4 and P-2-AM had only little or no effect, whereas DAM and MINA released important amounts of ^{32}P from the large molecules.

The AE and ChE reactivating potencies of some oximes. After incubation with 0·9 per cent NaCl in Tris-buffer of serum from rats injected with sarin the AE-activity was 35 per cent of that in similarly incubated serum from untreated rats. Incubation with DAM or MINA (0·5 mM) resulted in a significant reactivation of the AE-activity, whereas incubation with TMB-4 and P-2-AM did not (Table 2). This difference between DAM and MINA on the one hand and TMB-4 and P-2-AM on the other hand corresponds with that seen in the Sephadex experiments. The ChE reactivating potencies of the oximes, in contrast, did not concur with their ^{32}P-releasing properties: TMB-4, P-2-AM and MINA partially reactivated the ChE-activity, whereas DAM did not; P-2-AM was a significantly more effective reactivator than MINA.

Spontaneous reactivation of the plasma AE and ChE in the rat body. As illustrated in Table 2 the AE-activity was 1709 ± 191 (4) μl CO_2/ml/hr and the ChE-activity 360 ± 31 (4) μl CO_2/ml/hr in serum from blood taken from rats 10 min after the injection of 50 μg/kg of sarin. These values are somewhat lower than the corresponding values in plasma from blood 1 hr after the injection of 50 μg/kg of sarin, as presented

TABLE 2. THE AE- AND ChE-ACTIVITIES IN SERUM FROM RATS BLED 10 min AFTER THE i.v. INJECTION OF 50 μg/kg OF SARIN

	Serum from rats after injection of sarin					Serum from
	Control	TMB-4	P-2-AM	MINA	DAM	untreated rats
AE μl CO_2/ml/hr	1709 ± 191	1929 ± 223	2109 ± 309	2890 ± 217	3178 ± 220	4911 ± 226
percentage	35	39	43	59	65	
ChE μl CO_2/ml/hr	360 ± 31	776 ± 42	902 ± 47	597 ± 36	406 ± 34	1229 ± 75
percentage	29	63	73	49	33	

The serum was incubated during 18 min with a Tris-buffer containing either 0·9 per cent NaCl (control) or one of the oximes in a final concentration of 0·5 mM. Thereafter the enzyme activities were measured. These are expressed as μl CO_2/ml/hr (means of four determinations in duplicate ± S.E.M.) and as percentages of the mean simultaneously determined enzyme activities (means of seven determinations in duplicate ± S.E.M.) of serum from rats not injected with sarin.

in Table 2 of the preceding paper[1] (AE-activity 2492 ± 246 (9) μl CO_2/ml/hr, ChE-activity 404 ± 67 (9) μl CO_2/ml/hr).

In order to establish whether these differences could be attributed to the differences in the times of bleeding, the AE- and ChE-activities in serum from blood taken 10 and 60 min after the injection of 50 μg/kg of sarin were compared in simultaneously performed experiments. Ten min after the injection of sarin the AE- and ChE-activities were 1704 ± 236·3 (6) and 347 ± 32·2 (6) μl CO_2/ml/hr, respectively. After 60 min the AE-activity had risen significantly to 2820 ± 277 (7) and the ChE-activity to 493 ± 44·1 (7) μl CO_2/ml/hr. This suggests that the above-mentioned differences are due to a spontaneous partial reactivation of the enzyme activities in the body of the rat during the time interval between 10 and 60 min after the sarin injection.

DISCUSSION

Sephadex filtration of serum from rats after injection of ^{32}P-sarin demonstrated that part of the radioactive material present in the serum was attached to molecules with a molecular weight above 10,000. Incubation with oximes produced a decrease in the amounts of ^{32}P attached to the large molecules and an increase in those appearing in the micromolecular fractions. Apparently part of the ^{32}P was released by the oximes and DAM and MINA were much more effective than P-2-AM and TMB-4.

Subsequent determinations of the AE- and ChE-activities of samples of serum from rats after injection of sarin, which had been treated as much as possible in the same way as the samples submitted to Sephadex filtration, revealed that the AE-reactivating —in contrast to the ChE-reactivating—potencies of the oximes concurred with their radioactivity-releasing properties as measured by Sephadex filtration. This supports the idea that the serum proteins to which the ^{32}P was found to be attached in rats after injection of ^{32}P-sarin were identical with AE. The same conclusion was drawn earlier from the observation that pretreatment of rats with TOCP, an irreversible blocker of plasma AE in this species, strongly decreases the sarin-binding capacity of the rat plasma proteins.[2, 5]

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REFERENCES

1. R. L. POLAK and E. M. COHEN, *Biochem. Pharmac.*, **19**, 865 (1970).
2. R. L. POLAK and E. M. COHEN, *Biochem. Pharmac.* **18**, 813 (1969).
3. P. J. CHRISTEN and E. M. COHEN, *Abstracts Sixth Meeting of the Federation of European Biochemical Societies*, Madrid 7–11 April 1969, abstract 736, p. 235.
4. B. L. WELCH, *Biometr.* **34**, 18 (1947).
5. D. K. MYERS, *Biochim. biophys. Acta* **34**, 555 (1959).