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THE FATE OF TABUN IN ATROPINE AND ATROPINE-OXIME TREATED RATS AND MICE

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Abstract—The influence of TMB-4 and P2S on the metabolism and distribution of intravenously injected ^{32}P-labelled Tabun has been studied in atropinized rats and mice by means of whole animal autoradiography, quantitative determination of ^{32}P and paper chromatography. The largest part of the radioactivity was found in urine; smaller parts were found in faeces and traces in the expired air. In the presence of oximes the amount of ^{32}P excreted increased about 1·5 times. After 24 hr ^{32}P was found in decreasing concentrations in lung, kidney, skeletal bone, liver, heart, skeletal muscle and brain. In the presence of P2S the amount of ^{32}P in these tissues decreased to about 50%, while the decrease after TMB-4 was only 10–20% compared with untreated animals. The main metabolite of Tabun was dimethylamino ethoxy hydroxy phosphine oxide. Traces of ethoxy dihydroxy phosphine oxide were noticed in some experiments. In contrast to Tabun, injection of hydrolyzed Tabun produced no accumulation of ^{32}P in the lung.

CERTAIN oximes have been shown to possess an antidotal effect against dimethylamido ethoxphosphoryl cyanide (Tabun) if given together with atropin.[1, 2] The antidotal effect may depend on three facts: shielding of cholinesterase from the inhibitor, reactivation of the phosphorylated enzyme and reaction between the oxime and the inhibitor resulting in nontoxic products. These effects have been demonstrated *in vitro*.[3, 4] Reactivation and protection have also been shown *in vivo*.[5] The present investigation has been undertaken in order to study the possible influence of oximes on the metabolism and distribution of Tabun. Two oximes were chosen for study: N,N′-trimethylene bis (pyridinium-4-aldoxime) dibromide (TMB-4) and N-methylpyridinium-2-aldoxime methane sulphonate (P2S). They represent a good antidote and potent reactivator and a less effective antidote without reactivating power respectively.

MATERIAL AND METHOD

Materials

^{32}P-labelled Tabun (activity approximately 100 mc/mg Tabun) was synthesized as described in a previous paper.[6] Dimethylamino ethoxy hydroxy phosphine oxide (DEHPO), ethoxy dihydroxy phosphine oxide (EDHPO) and dimethylamino dihydroxy phosphine oxide (DDHPO) were synthesized as sodium salts from their corresponding phosphorylchlorides according to methods described elsewhere.[7] All other compounds were commercial products. The oximes were recrystallized before use.

Methods

Three groups, each composed of six rats, were injected with atropine, 10 mg/kg i.m. Two groups received either P2S (50 mg/kg i.m.) or TMB-4 (30 mg/kg i.m.), 10 min after atropine. All three groups received Tabun (0·08 mg/kg i.v.) 25 min after atropinization. The rats were kept in metabolic cages and obtained water and food *ad libitum*. Urine and faeces were collected at certain time intervals. Urine, voided directly into chilled test tubes, was collected within a few minutes after the injection of Tabun for chromatography and determination of anticholinesterase activity. Blood samples were taken after decapitation of heparinized animals. For the collection of bile the common bile duct was cannulated with a polyethylene catheter under ether anaesthesia twelve hours before the injection of Tabun. The rats were allowed to recover and had free access to saline solution. The bile was collected in chilled test tubes and used for chromatography and measurements of total radioactivity and anticholinesterase activity.

Tissues from rats killed by decapitation were immediately excised, washed with 0·9% NaCl, blotted and weighed. Except for skeletal muscle and bone the whole organs were used. They were wet-ashed with concentrated sulphuric acid and nitric acid. Bones were dry-ashed in a furnace at 850°–900°. Aliquots of all samples were diluted to fixed volume with phosphate solution prior to radioassay. Radioactivity was measured with a Geiger–Müller liquid counter. It was verified by addition of known amounts of labelled Tabun before and after ashing that no radioactivity was lost during ashing and also that the presence of urine or blood did not reduce the radioactivity. The results were calculated on the basis of measurements obtained the same day with aliquots of the injected Tabun solution

For measurements of radioactivity in the expired air rats were kept in large desiccators and an air stream of about 1 l/min was passed through the vessel. In order to collect acid products and organophosphorus compounds, the expired air was allowed to pass through a filter consisting of a 10 mm layer of carbon (2·5 g, no 0:4, Pittsburgh U.S.A.) impregnated with copper carbonate, ammonium dichromate, silver nitrate and ammonium carbonate. The radioactivity of the carbon was measured on planchettes before and after the experiment. Control experiments with ^{32}P-solutions showed that the counting efficiency of ^{32}P was lowered about 50% in the presence of 2·5 g of carbon.

Cholinesterase activity and phosphorylphosphatase activity was measured with an electrometric method[8] at pH 8·0 and 25°. The tissues were homogenized in veronal buffer with a Potter–Elvehjem homogenizer. The final concentration of acetylcholine iodide was $7{\cdot}3 \times 10^{-3}$ M and that of Tabun $5{\cdot}0 \times 10^{-3}$ M. Control bile lowered the cholinesterase activity of a standard cholinesterase preparation (purified plasma cholinesterase) depending upon the amount of bile present, but the percent inhibition produced by Tabun was not influenced.

Paper chromatography was performed on Whatman No. 1 paper washed with hydrochloric acid. The solvent was *n*-propanol–ammonia–water (6 : 5 : 1). This solvent system was chosen after preliminary experiments with acid, neutral and alkaline solvent systems. Tabun was only partly recovered when chromatographed, due to the volatility of the compound. The chromatograms were submitted to autoradiography, and the reference substances were located by a modification of the molybdenum method for phosphate esters.[9, 10]

For whole body autoradiography mice (weight 20 g, female) received atropine s.c. (10 mg/kg) 25 min before an i.v. injection of 0·23 or 1·04 mg/kg of Tabun (1 and 4·5 LD_{50} respectively). The specific activity on the day of the experiment was about 25 mc/g of Tabun. In one series of experiments TMB-4 (10 mg/kg, i.v.) was given 10 min before Tabun. Some animals received corresponding amounts of hydrolyzed Tabun (Tabun left for three days in distilled water at room temperature). Four animals were pregnant. The animals were frozen by immersion in carbon dioxide and acetone either immediately after the injection of the labelled compound or hydrolysate or after 20, 60, 240 min and 24 hr. The autoradiographic technique has been described in detail previously.[11, 24]

RESULTS

Metabolites of Tabun

No intact Tabun (limit of the method 3×10^{-7} M Tabun) was found in the urine of rats after i.v. injection of ^{32}P-labelled Tabun as judged from incubation experiments with a cholinesterase preparation. Autoradiochromatograms of the urine demonstrated one major metabolite with the same R_f-value (0·70–0·76) as an authentic specimen of DEHPO dissolved in urine (Fig. 1). This compound was found 2 min after the injection of labelled Tabun and was excreted during at least 3 days in decreasing amounts. The presence of minute amounts of another metabolite with the same R_f-value (0·31–0·38) as a synthetic specimen of EDHPO was observed in some urine samples collected after 2–3 days. The same metabolites were found in the urine from both P2S and TMB-4 treated animals. Bile collected during 30 min after the injection of Tabun contained no Tabun as judged from incubation experiments with a cholinesterase preparation. In bile collected after 3 hr an unknown spot with the R_f value of 0·67 was seen. This latter spot was not found in samples of Tabun incubated *in vitro* with bile for 3 hr. No DEHPO was found in the bile.

Distribution and excretion

The amount of ^{32}P derived from i.v. injected Tabun and found in the tissues of atropinised rats is seen in Table 1. After 24 hr lung, kidney, liver and bone retain the largest parts of the radioactivity, while smaller amounts are found in brain, heart and skeletal muscle. About 15% of the radioactivity is excreted in the urine during the first 24 hr. Much less was found in bile and faeces (0·3 and 0·9%, Table 2). After 6 days the concentration of ^{32}P had declined markedly in the organs analyzed except in brain and bone where the radioactivity had roughly doubled. Autoradiography shows that the radioactivity of the kidneys reached its maximum value within 20 min. High amounts of radioactivity are found in the liver and they increase during the first 4 hr (cf. Figs 3 and 4). Practically no activity is seen in the bile, while the intestinal mucosa is rather active 20 min after the Tabun injection and later on the intestinal contents also seem to be radioactive. Autoradiograms obtained from pregnant animals showed that ^{32}P had passed through the placental barrier 20 min after the injection of Tabun. The radioactivity is localized mainly in the bones of the foetus. In the ovaries radioactivity is found in the corpora lutea (Fig. 2).

Despite the high concentration of ^{32}P in the lungs compared with other tissues only small amounts of radioactivity were found in the expired air. During the first 90 min after the injection of Tabun only 0·05–0·13% of the injected radioactivity was

TABLE 1.

Tissue	Time after injection of Tabun	A + T		A + P2S + T		A + TMB-4 + T	
		Injected amount (%)	μg ^{32}P/g wet wt. $\times 10^3$	Injected amount (%)	μg/^{32}P/g wet wt. $\times 10^3$	Injected amount (%)	μg ^{32}P/g wet wt. $\times 10^3$
Liver	24 hr	2·9 ± 0·4	10·1	1·8 ± 0·2	5·6	2·6	8·2
	6 days	1·2 ± 0·4	4·2	0·9	3·4	1·5 ± 0·1	6·0
Brain	24 hr	0·09 ± 0·02	2·0	0·05 ± 0·01	1·0	0·07 ± 0·01	1·6
	6 days	0·17 ± 0·1	3·9	0·08	1·3	0·14 ± 0·02	2·5
Heart	24 hr	0·14 ± 0·02	6·7	0·08 ± 0·01	4·6	0·11 ± 0·01	6·2
	6 days	0·07 ± 0·03	2·7	0·05 ± 0·000	2·3	0·08 ± 0·00	3·8
Kidney	24 hr	3·4 ± 0·4	72				
	6 days	0·46 ± 0·04	9·3	0·24 ± 0·01	5·3	0·45 ± 0·02	9·1
Lung	24 hr	5·4 ± 0·3	165				
	6 days	0·59	17·7	0·52 ± 0·07	16·0	0·65 ± 0·02	18·9
Skeletal muscle	24 hr		2·0		1·0		1·6
	6 days		1·5		1·5		2·2
Skeletal bone	24 hr		18·6		8·3		14·7
	6 days		30·6		20·0		33·4

Amount of Tabun derived ^{32}P found in tissues and bone from rats. A = 10 mg/kg atropine i.m., T = 0·08 mg/kg Tabun i.v., P2S = 50 mg/kg i.m., TMB-4 = 30 mg/kg TMB-4 i.m.

The total weight of skeletal muscle and bone was not estimated.

FIG. 1. Autoradiogram of a sample of urine from a rat injected i.v. with ^{32}P-labelled Tabun. S = injected solution diluted 1 : 10 with urine, urine, urine rat 3 = urine taken 2 min after Tabun injection. DEHPO, DDHPO and EDHPO are reference substances.

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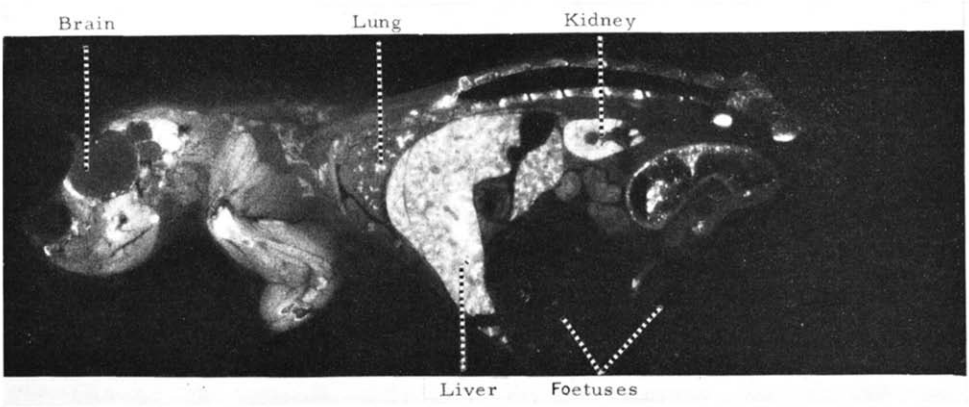


FIG. 2. Autoradiogram from a pregnant mouse 4 hr after injection i.v. of ^{32}P-labelled Tabun. The animal had also received TMB-4. Notice the high amount of ^{32}P in the bronchi and in the bones of foetus (light areas).

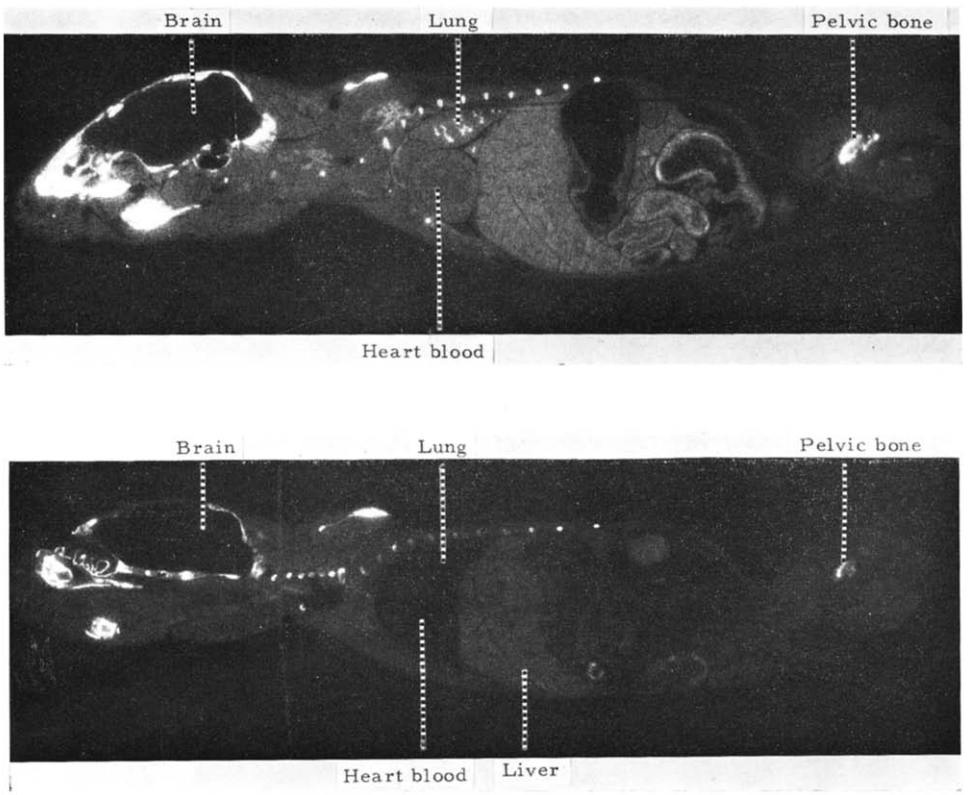
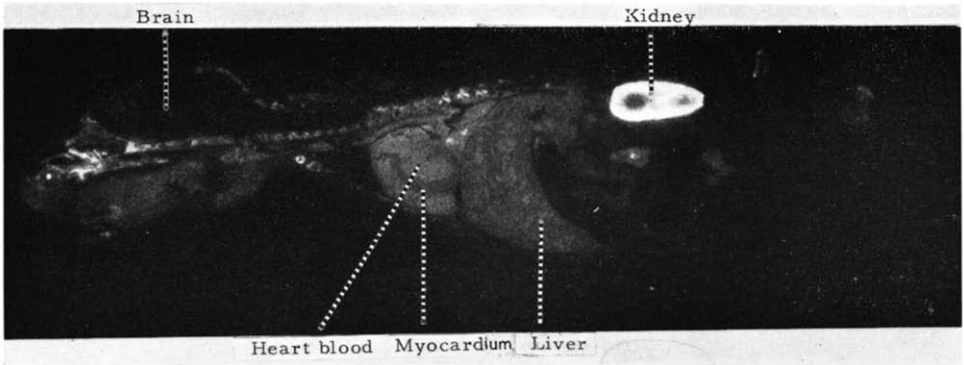


FIG. 3. Autoradiograms from mice 24 hr after injection of ^{32}P-labelled Tabun (upper one) and hydrolyzed ^{32}P-labelled Tabun (lower one). The blood concentration of ^{32}P diminishes more rapidly after the injection of the hydrolysed compound. Notice the difference in the amount of ^{32}P in the lungs.



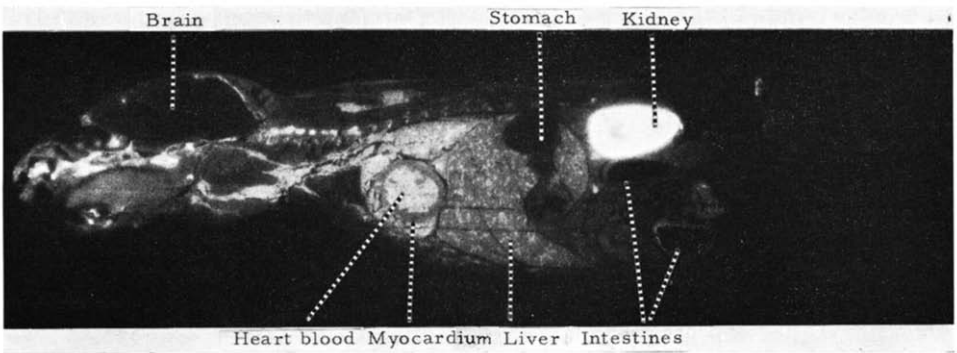


FIG. 4. Autoradiograms from two mice 20 min after injection i.v. of ^{32}P-labelled Tabun. The upper one received atropine and the lower one atropine and TMB-4 before injection of Tabun. No significant difference in the distribution pattern can be seen.

recovered. This means that less than 2·3% is excreted by this route during 24 hr if it is assumed that the excretion is linear with time. At the end of the experiment the amount of ^{32}P in the blood of these rats was about 12% of the injected amount. The high radioactivity in the lungs is seen also on the autoradiograms. While after 20 min (Fig. 4, upper) the radioactivity in the lungs is more uniform, this activity seems to be located mainly in the bronchial walls after 4 hr (Fig. 3, upper).

Injection of hydrolysed Tabun showed that the radioactivity in the blood diminishes more rapidly and that of the bone increases more rapidly than after Tabun. In addition the ^{32}P amount of liver seems to be higher when the hydrolysate is injected. Low concentration of ^{32}P was seen in the lungs (Fig. 3, lower).

The quantitative determination of ^{32}P in the tissues following injection i.m. of P2S shows (Tables 1 and 2) that 24 hr after the injection of Tabun the percent ^{32}P

TABLE 2.

	Time after Tabun injection	A + T	A + P2S + T	A + TMB-4 + T
Urine	1 hr	0·7	1·4	1·8
	24 hr	14·5	19·5	21·4
	48 hr	24·0	30·1	36·3
	6 days	32·0	41·1	51·3
		(25·4–33·0)	(34·2–59·7)	(41·9–52·9)
Faeces	24 hr	0·9	0·5	0·8
	3 days	4·5	3·5	4·6
Bile	24 hr	0·3	0·2	0·4

Percentage of Tabun derived ^{32}P found in excretion products and bile from rats. The values given are cumulative. A = 10 mg/kg atropine i.m., T = 0·08 mg/kg Tabun i.v., P2S = 50 mg/kg P2S i.m., TMB-4 = 30 mg/kg TMB-4 i.m. Values in brackets show highest and lowest amount of ^{32}P in urine for three rats.

retained in the organs is about 50% lower than in the controls. This effect is not as obvious after TMB-4. About 50% more ^{32}P is found in the urine after injection of P2S or TMB-4 than in the controls. The autoradiograms show no fundamental difference in the distribution pattern when TMB-4 is given before Tabun (Fig. 4).

Enzyme activities

In order to see whether there was any correlation in the amount of radioactivity found in different tissues and the occurrence of esterases known to react with or to destroy Tabun,[12] cholinesterase and phosphorylphosphatase ('Tabunase') were determined in different tissues. The results seen in Table 3 show that there is no correlation between enzyme activities and radioactivity.

DISCUSSION

Similar to the results obtained from experiments with labelled DFP[13] the main route of excretion of ^{32}P after administration of Tabun was found to be via the kidneys. A small amount of radioactivity was found in faeces, and is probably due to secretion from intestinal mucosa, as autoradiograms demonstrate radioactivity in the mucosa and later in the intestinal contents and the amount of ^{32}P in bile is extremely low.

Tissues known to have high amounts of cholinesterase, such as muscle and brain, retain very little ^{32}P, whereas other organs such as liver and kidney bind much ^{32}P. Metabolites may be bound, as these tissues contain a high amount of the detoxificating enzyme phosphorylphosphatase. A more general phosphorylation of proteins may occur in tissues, as Murachi has shown that several proteins are able to bind DFP *in vitro*.[14] Also it is known that several esterases in the liver are phosphorylated by DFP.[15]

TABLE 3.

Tissue	'Tabunase'	Cholinesterase
Brain	117 ± 38	419 ± 19
Lung	80 ± 16	176 ± 9
Liver	331 ± 37	112 ± 9
Kidney	235 ± 19	69 ± 5
Heart	139 ± 28	309 ± 38
Skeletal muscle	112 ± 52	64 ± 18

Relative enzyme activities per g wet wt. Mean of three measurements at pH 8·0 and 25°C, using 5×10^{-3} M Tabun, $7{\cdot}3 \times 10^{-3}$ M acetylcholine iodide as substrates.

Both TMB-4 and P2S increase the amount of radioactivity excreted in the urine, but P2S is, in contrast to TMB-4, a very poor reactivator of Tabun inhibited cholinesterases.[3, 4, 16] The fact that both oximes increase the amount of excreted ^{32}P thus excludes reactivation of cholinesterase as the only explanation for this observation. Perhaps P2S protects better against phosphorylation of other compounds. It probably does not protect any cholinesterases as it has been shown that it is not able to protect blood cholinesterases *in vivo*.[5] However, *in vitro* experiments show that both oximes influence the breakdown of Tabun[3, 7] and they may also influence the excretion of the metabolites.

Tabun is rapidly metabolized in the body as judged from the occurrence of dimethylamino ethoxy hydroxy phosphine oxide (DEHPO) in urine within 2 min after the injection i.v. of Tabun and from the rapid incorporation of ^{32}P in bone. This is to be expected as it has long been known that there are enzymes in the body able to split Tabun to DEHPO.[12] (Spontaneous hydrolysis of Tabun can only account for less than 1% in 24 hr[17]). The increasing radioactivity of bone and brain during 6 days after the Tabun injection may indicate a further breakdown of DEHPO to orthophosphate.

The oximes had no influence upon the type of metabolite formed. Previous *in vitro* experiments have shown[7] that about 30% of the dimethylamino group of Tabun is split off at physiological pH and 25° in the presence of a 100-fold excess of oxime within 140 hr. Although the body temperature is higher and an approximate 500-fold initial excess of oxime was used in the *in vivo* experiments, no evidence for the occurrence of this reaction *in vivo* was obtained. This may be due to the fact that the oxime is rapidly excreted.[18] Only in 2 experiments out of 12, spots with the same R_f value as ethoxy dihydroxy phosphine oxide, the reaction product in the deamination of Tabun, were noticed. Any formation of a stable reaction product between oxime and Tabun could not be demonstrated in our experiments which is to be expected as phosphorylated oximes are rather unstable compounds.[7, 19]

The large amount of radioactivity observed in the lungs following Tabun injection is similar to observations with Sarin and DFP.[20, 21] Obviously the radioactivity is trapped in the lung tissue, as little ^{32}P was found in expired air in our experiments. This accumulation seems to be rather specific for the intact cholinesterase inhibitor, as it is not observed after the injection of diisopropylmethyl phosphate, a compound not able to react with cholinesterases[20] or after hydrolysates of Tabun. It has recently been shown that the insecticide ethyl *p*-nitrophenyl thionobenzene phosphonate (EPN), given a few hours before ^{32}P-labelled Sarin, prevents the incorporation of ^{32}P in the lung.[22] It has also been shown that Sarin enhances the incorporation of ^{32}P derived from diisopropylmethyl phosphate in the lung.[20] A possible explanation for these findings is that the cholinesterase inhibitor causes profound changes in the haemodynamics of the lung (e.g. pulmonary congestion[23]) leading to trapping of ^{32}P.

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