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THE INFLUENCE OF DFP, ATROPINE AND PYRIDINIUM ALDOXIMES ON THE RATE OF CLEARANCE OF DIISOPROPYL PHOSPHATE ($DI^{32}P$) FROM THE MOUSE CIRCULATORY SYSTEM

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Abstract—$DI^{32}P$, the hydrolytic product of radioactive di-isopropyl phosphorofluoridate ($DF^{32}P$), when injected to mice, is rapidly excreted. If it is mixed with non-radioactive DFP or eserine (physostigmine) and injected, there is an increase in the radioactivity content in the liver, kidney, lung and blood. Since $DI^{32}P$ is not actively transported in the cell it is considered that the DFP- or eserine-induced accumulation of $DI^{32}P$ in the tissues is due to the depression of the circulatory rate. Atropine rapidly corrects this condition bringing the blood content of $DI^{32}P$ to normal levels. The pyridinium aldoximes, *N*-methyl pyridinium-2-aldoxime chloride (PAM), *N,N'*-trimethylenebis (pyridinium-4-aldoxime) dichloride (TMB-4) and *N,N'*-oxymethylenebis (pyridinium-4-aldoxime) dichloride (Toxogonin) which are the common antidotes against organophospate poisoning have no perceptible effect in restoring the normal rate of clearance of $DI^{32}P$ during the period of observation. It is likely that the potentiating effect of atropine on the antidotal action of oximes is exerted by improving the circulation rate and thus the turn-over of the oximes in vital regions. When a mixture of TMB-4 and atropine is injected there is a higher concentration of the oxime in blood at 30 min than when TMB-4 is injected alone. A similar effect is observed in DFP-treated animals. These effects are observed to a lesser extent with PAM.

PYRIDINIUM aldoximes are powerful *in vitro* reactivators of phosphorylated acetylcholinesterase (AChE) formed by certain organophosphorus esters. However, they afford only limited antidotal protection to experimental animals against these compounds, being unable to raise the LD_{50} by more than a few multiples.[1, 2] On the other hand, if atropine is also administered the antidotal effect is increased several-fold, to a level which is greater than that attributable to the summation of the individual effect of the oxime and atropine. This potentiating effect of atropine is not clearly understood.

Quaternary oximes being ionized compounds do not easily penetrate the blood-brain barrier,[3, 4] and there is a lack of correlation in the central nervous system between the antidotal effect of an oxime (with atropine) and its reactivating potency.[5] It has been suggested that certain parts of the brain may be more accessible to oximes than others[6–10] and that the external or functional AChE may be preferentially involved.[5]

A factor which may be of relevance in these transactions is the possibility that atropine may have a function other than the anti-muscarinic effect conventionally

ascribed to it. For example, it is well-known that acetylcholine (ACh) and atropine have profound effects on membrane permeability. Atropine may thus influence penetration or prevent dissipation of the oximes either by directly modifying the cell-membrane or indirectly by improving the rate of circulation in vital regions of the body.

In the present work data are presented to show that DFP and eserine cause a decrease in the rate of clearance of $DI^{32}P$, the hydrolytic product of $DF^{32}P$, from mouse tissues. Atropine corrects this condition, presumably by improving the circulatory rate depressed by the anti-cholinesterases. Pyridinium aldoximes do not seem to have an effect on this condition.

$DI^{32}P$ used in these studies as an indicator substance is in itself an innocuous compound which does not bind to the tissues[11] and is not further metabolized in the system.[12]

MATERIALS AND METHODS

Unlabelled DFP was purchased from Sigma Chemical Co., St. Louis, U.S.A. A 10% (w/w) solution was prepared in anhydrous propylene glycol and preserved at −16° and quantities were weighed and diluted before experiments. $DF^{32}P$ (300 mc/g) was obtained from Radiochemical Centre, Amersham, England. It contained varying quantities of un-extractable impurity (usually 7–15%) which consisted of $DI^{32}P$, $^{32}P_i$ (radioactive inorganic phosphate) and $MI^{32}P$ (radioactive monoisopropyl phosphate). It might also have contained small amounts of other impurities, since in the method used for analysis[13] the value for $MI^{32}P$ was obtained by difference. $DF^{32}P$ was also preserved at −16° as a stock solution in propylene glycol which contained 1·85 mg (10 μmole) per gram.

$DI^{32}P$ was prepared according to the method described.[13] Briefly, solutions for injection were prepared as follows: a mixture of 2·00 g of $DF^{32}P$ solution and 150 mg of 10% DFP was dissolved in 25 ml of 0·2 N NaOH and allowed to remain at room temperature for 1 hr. It was diluted to 50 ml after the addition of 5 ml of 10 N H_2SO_4 and extracted twice with 50 ml portions of a 1:1 mixture of isobutanol and benzene. The solvent phase was extracted twice respectively with 10 and 5 ml of 0·2 N NaOH. The alkaline phase was exactly neutralized (phenolphthalein) with 1 N HCl and the volume adjusted to 20 ml. The solution is isotonic and if injected at 4 ml/kg will give approximately 3·6 mg of DIP/kg of mouse.

Atropine and eserine were used as the sulphates. These and other chemicals were obtained from the usual commercial sources. PAM, TMB-4 and Toxogonin were obtained through the Research Institute of National Defence, Department 1, Sundbyberg 4, Sweden. Atropine was always administered intraperitoneally at 17·4 mg (0·05 m-mole)/kg and the oximes at 0·1 m-mole/kg by the same route. DFP, $DI^{32}P$ and eserine were by the subcutaneous route. All solutions were freshly prepared before the experiments except $DI^{32}P$ which was stable at 0–4° for several days.

Male albino mice of 20–25 g were used in all experiments. For any series, animals of uniform weight (±1 g) were chosen. DFP was administered at 3·4 mg/kg (LD_{50} = 3·8 mg).[2, 14] At this dose the animals showed the symptoms of poisoning but none died. Unless otherwise stated DFP was mixed with $DI^{32}P$. Eserine was similarly

mixed with DI^{32}P at 0·6 mg/kg (LD_{50} = 0·75 mg).[15] The animals were killed by striking the head on the edge of the table and quickly clipping off the head. Blood was collected in cups containing a drop of heparin. One ml was diluted to 5 or 10 ml for radioactivity determinations. The tissues were quickly washed in saline to remove residual blood and homogenized with water in a Potter–Elvehjem type homogenizer. Aliquots were pipetted into small glass cups in triplicate and dried at 110°. Radioactivity was determined in an automatic fraction changer (LKB-Produkter AB, Stockholm) fitted with an end-window TGC-2 Geiger tube. Tissue slice experiments were carried out at 37° in Krebs-bicarbonate buffer saturated with oxygen. Slices approximately 0·5 mm thick, were cut free-hand by a razor blade.

Other details are as in previous papers[2, 14, 16] or are given in appropriate places in the text. Pooled samples from 2 to 5 animals were usually worked up. Throughout the text the values for radioactivity have been calculated as mμg of DI^{32}P-derived P from a standard curve and expressed as for 1 g of tissue or 1 ml of blood.

RESULTS

Preliminary experiments with DI^{32}P. When DI^{32}P was injected to mice, the trichloroacetic acid-soluble portion of the liver, kidney, lung and blood contained only DI^{32}P as determined by the method described.[13] This confirms that there is no further degradation of DI^{32}P in the animal system.[12] The tissues also did not contain any bound-^{32}P thereby corroborating the results of previous workers.[11, 12] When DI^{32}P was incubated at 37° with tissue homogenates there was no formation of MI^{32}P or $^{32}P_i$. There was no binding of radioactivity to the tissue. After injection there was no measureable amount of DI^{32}P in the brain, showing that it does not easily penetrate the barrier.

When DF^{32}P was injected, the acid-soluble part of the tissues contained, in addition to DI^{32}P, some MI^{32}P and $^{32}P_i$. These were presumably derived from the smal amount of impurity in the DF^{32}P. Whereas DI^{32}P disappeared from the organs rapidly, MI^{32}P and $^{32}P_i$ persisted for a much longer time.

Uptake of DI^{32}P in tissue slices in vitro. Several experiments were performed with liver, kidney and heart slices for studying the uptake of DI^{32}P in these tissues. The oxygenated Krebs-bicarbonate medium contained DI^{32}P at 10^{-6} M and in addition the following in various combinations: DFP, acetylcholine, eserine and atropine at 3 concentrations of 10^{-4}, 10^{-5} and 10^{-6} M. The slice to medium (S/M) ratio measured at intervals from 5 min to 2 hr showed that it rose from 0·2 at 15 min to about 0·5 or 0·6 in the course of an hour. The maximum value of 0·7 was reached in 2 hr after which there was no further rise. This shows that DI^{32}P is not actively transported in the cell but that it penetrates by simple diffusion. Assuming the extra-cellular space as approximately 30% (ref. 17), the S/M ratio of 0·7 indicates that DI^{32}P penetrates inside the cell to an appreciable extent. None of the adjuvants had a significant effect on the rate of DI^{32}P penetration in the slices. The details of these negative results are not given.

Inhibitory effect of DFP and eserine on the rate of clearance of DI^{32}P from tissues. When DI^{32}P was injected to mice it was rapidly excreted from the tissues and blood as indicated by its rate of disappearance (Table 1). Only the values for liver, kidney and blood are given. Lung tissue gave similar results. Very little was taken up in the brain.

When $DI^{32}P$ was mixed with unlabelled DFP there was a higher concentration of radioactivity in the tissues. All the differences are statistically significant ($P<0.05$). McPhail and Adie[18] observed that radioactive diisopropyl methyl phosphate which is not an anti-cholinesterase, when injected to rabbits did not result in a high concentration in the lung. However, when it was in combination with non-radioactive sarin there was a much higher concentration.

TABLE 1. THE EFFECT OF DFP AND ESERINE ON THE CLEARANCE RATE OF $DI^{32}P$ FROM MOUSE TISSUES

Treatment	Tissue	mμg of $DI^{32}P$-derived P in 1 g of tissue or 1 ml of blood, with S.D. and no. of trials			
		0·5 hr	1 hr	2 hr	4 hr
$DI^{32}P$ only	Liver	557 ± 126 (8)	182 ± 42 (5)	44 ± 17 (10)	25 ± 5 (4)
$DI^{32}P$ + DFP	Liver	828 ± 98 (8)	259 ± 97 (7)	83 ± 22 (7)	42 ± 23 (3)
$DI^{32}P$ + eserine	Liver		279 ± 60 (4)	55 ± 3 (2)	
$DI^{32}P$ only	Kidney	1384 ± 131 (6)	317 ± 131 (5)	45 ± 4 (5)	40 ± 9 (3)
$DI^{32}P$ + DFP	Kidney	2232 ± 692 (8)	1136 ± 435 (7)	165 ± 68 (4)	133 ± 128 (4)
$DI^{32}P$ + eserine	Kidney		455 ± 131 (3)	77 ± 1 (2)	
$DI^{32}P$ only	Blood	366 ± 114 (3)	55 ± 17 (9)	17 ± 4 (7)	14 ± 4 (3)
$DI^{32}P$ + DFP	Blood	587 ± 198 (4)	155 ± 36 (4)	34 ± 9 (5)	27 ± 19 (4)
$DI^{32}P$ + eserine	Blood		134 ± 19 (4)	24 ± 2 (2)	

Groups of 2–4 mice were injected either $DI^{32}P$ alone at 3·6 mg/kg or mixed with DFP at 3·4 mg/kg or eserine at 0·6 mg/kg. They were sacrificed at the intervals indicated and the pooled tissues were analysed as described under Methods. The differences between the $DI^{32}P$ values and those with the anti-cholinesterases are statistically significant in every case ($P< 0.05$).

Eserine mixed with $DI^{32}P$ gave similar results (Table 1). The difference in the effect of $DI^{32}P$ alone and a combination of $DI^{32}P$ and DFP is not probably due to physico-chemical effects. An extra amount of unlabelled DIP added to the $DI^{32}P$ in the control to compensate for the quantity derived from the DFP did not alter the results.

Effect of DFP on the transport ATPase-system in vivo. It has already been mentioned that $DI^{32}P$ is not actively transported in the liver, kidney and heart slices. It is not likely that the effect of anticholinesterases in causing a higher concentration of $DI^{32}P$ in tissues is due to any disturbance in the transport system operating in the cells.

Phosphates are implicated as taking part in the Na^+ and K^+ transport ATPase system. Hokin and Yoda[19] found that transport-ATPase system is irreversibly inhibited by DFP *in vitro*. They had used DFP concentrations of 10^{-3} M and above. To see whether DFP had any effect on the transport-ATPase *in vivo*, mice were injected DFP at 3·4 mg/kg at which they showed characteristic symptoms of organophosphate poisoning. There were no deaths. ATPase, both with 10^{-4} M strophanthin G and without, were determined in the kidney, brain and liver. As seen from results in Table 2, almost identical values were obtained for transport ATPase in the control animals as well as in animals treated with DFP. The changes found in the $DI^{32}P$ concentrations of tissues as a result of DFP are not, therefore, due to any direct or indirect interference with the transport-ATPase system by the organophosphate.

When strophanthin G (ouabain) was injected to mice at 8 mg/kg i.p. (LD_{50} = 8–13 mg)[15] followed 15 min later by $DI^{32}P$ there was no difference in the $DI^{32}P$ content of the organs from the controls without ouabain.

Effect of atropine and oximes on the anti-cholinesterase-induced accumulation of $DI^{32}P$ in blood. When animals were pre-treated with atropine and then injected with $DI^{32}P$ + DFP there was no accumulation of $DI^{32}P$ in the tissues. In Table 3 only the

TABLE 2. THE EFFECT OF INJECTING MICE WITH DFP ON THE TRANSPORT ATPASE CONTENT OF TISSUES

Treatment	Interval between DFP and sacrifice	Transport ATPase activity in μmole of P_i liberated in 1 hr by 1 g of tissue; with S.D. and no. of trials		
		Brain	Kidney	Liver
None	—	2773 ± 600 (6)	1328 ± 309 (6)	198 ± 48 (3)
DFP at 3·4 mg/kg	30 min	2808 ± 624 (5)		
DFP at 3·4 mg/kg	60 min		1302 ± 291 (6)	187 ± 32 (3)
DFP at 3·4 mg/kg	4 hr	2797 ± 412 (5)		

ATPase was determined by adding 1 ml of a 1:100 homogenate of the tissue in 0·01 M Tris-acetic acid buffer of pH 7·4 which contained 0·005 M EDTA to 9 ml of a 0·02 M imidazole-histidine buffer of pH 7·4 which contained the ingredients to give the following final concentrations: NaCl 100 mM, KCl 20 mM, ATP 3 mM, $MgCl_2$, 6 H_2O 3 mM, with or without ouabain at 10^{-4}M. Temperature of incubation 37°. 1 ml aliquots were withdrawn at 5-min intervals for determination of inorganic phosphate by the Martin and Doty method. From the straight-line graphs obtained the difference between the total and the ouabain-resistant ATPase activity was calculated.

TABLE 3. THE EFFECT OF ATROPINE AND PYRIDINIUM ALDOXIMES ON THE RATE OF CLEARANCE OF $DI^{32}P$ FROM THE CIRCULATORY SYSTEM OF THE MOUSE

No.	Treatment	mμg of $DI^{32}P$-derived P in 1 ml of blood, with S.D. and no. of trials		
		DFP		Eserine
		1 hr	2 hr	1 hr
1.	$DI^{32}P$ only	55 ± 17 (9)	17 ± 4 (7)	55 ± 17 (9)
2.	$DI^{32}P$ + anti-ChE	155 ± 36 (4)	34 ± 9 (5)	134 ± 19 (4)
3.	$DI^{32}P$ + anti-ChE + atropine (P)	53 ± 7 (3)	15 ± 2 (3)	89 ± 20 (4)
4.	$DI^{32}P$ + anti-ChE + atropine (T)	101 ± 20 (4)	25 ± 7 (3)	124 ± 24 (3)
5.	$DI^{32}P$ + anti-ChE + PAM (P)	145 ± 24 (2)	39 ± 13 (4)	109 ± 3 (2)
6.	$DI^{32}P$ + anti-ChE + PAM (T)	123 ± 26 (4)	52 ± 10 (3)	98 ± 13 (2)
7.	$DI^{32}P$ + anti-ChE + TMB-4 (P)	220 ± 79 (3)	31 ± 4 (4)	104 ± 6 (2)
8.	$DI^{32}P$ + anti-ChE + TMB-4 (T)	124 ± 20 (5)	38 ± 2 (3)	117 ± 12 (2)
9.	$DI^{32}P$ + anti-ChE + Toxogonin (P)	163 ± 50 (3)	30 ± 11 (4)	71 ± 13 (3)
10.	$DI^{32}P$ + anti-ChE + Toxogonin (T)	147 ± 16 (3)	21 ± 3 (4)	115 ± 32 (4)

Groups of 2–4 mice were injected either $DI^{32}P$ alone at 3·6 mg/kg or $DI^{32}P$ mixed with DFP (at 3·4 mg/kg) or eserine sulphate (0·6 mg/kg). The mice treated with the anti-cholinesterases received atropine (0·05 m-mole/kg) or oximes (0·1 m-mole/kg) either 15 min before (P) or 15 min after (T) the $DI^{32}P$ + anti-cholinesterase treatment. Other details are given under Methods. The $DI^{32}P$ content of liver and kidney also gave similar results, but only the values for blood are reported.

values for blood are given. There was a similar effect in the liver and kidney, the values for the latter being very variable due to the presence of tubular urine. If atropine was injected after $DI^{32}P$ + DFP the values were still very much lowered.

PAM and TMB-4 either given before or after $DI^{32}P$ + DFP did not have any effect on the $DI^{32}P$ content of the blood or tissues up to the period of observation, viz., 2 hr. Toxogonin seems to have a slight atropine-like effect, but before definite

conclusions can be drawn on the relative merits of the oximes, more experiments will have to be done to assess the significance of the data. The effect of Toxogonin is perceptible in the 2-hr values.

With the data at present available it is safe to conclude that atropine has an effect which is far superior to those of the oximes in restoring to normalcy a condition in which DFP induces the accumulation of $DI^{32}P$ in blood and tissues.

Atropine has a similar effect on eserine-induced accumulation of $DI^{32}P$ in blood. Here again the oximes do not have a perceptible effect. Toxogonin shows a slight influence when given prophylactically. Because of the rapid recovery of the animals from this reversible anti-cholinesterase, only values for the 1-hr period were determined.

The effect of DFP and atropine on the concentration of PAM and TMB-4 in mouse blood. The action of DFP in effecting a higher concentration of $DI^{32}P$ in tissues and circulation may be of practical significance if the concentration of oximes administered as antidotes is also similarly affected. In this case, however, it is more complicated since oximes and DFP mutually react chemically. Also oximes are probably transported actively and not merely by simple diffusion[21] like $DI^{32}P$.

However, to study the over-all effects, mice were injected either PAM or TMB-4, with or without atropine. In one series the animals had been pre-treated with DFP 1 hr before the administration of oximes. Previous work has[2] shown that there is very little free DFP in the system at this interval and dose. The mice were sacrificed at 30 min after the oximes and the blood-content of the oximes was determined spectrophotometrically according to Sundwall.[20] Experiments were not performed with Toxogonin as low readings were obtained for this oxime at the dosage employed.

From results in Table 4 it is seen that there is a highly significant, higher concentration of TMB-4 in animals which had been pre-treated with DFP. It is of considerable

TABLE 4. THE EFFECT OF DFP AND ATROPINE ON THE CONCENTRATION PAM AND TMB-4 IN MOUSE BLOOD

Treatment	mμmole of oxime in 1 ml of blood with S.D. and No. of trials	Significance (P)
PAM only	15·8 ± 4·1 (11)	
PAM, 1 hr after DFP	17·4 ± 5·3 (10)	> 0·1
PAM only	15·8 ± 4·1 (11)	
PAM + atropine	18·3 ± 9·2 (9)	> 0·1
PAM + atropine, 1 hr after DFP	29·8 ± 5·6 (7)	< 0·001
TMB-4 only	29·0 ± 4·2 (10)	
TMB-4, 1 hr after DFP	49·3 ± 7·6 (10)	< 0·001
TMB-4 only	29·0 ± 4·2 (10)	
TMB-4 + atropine	38·6 ± 6·6 (10)	< 0·001
TMB-4 + atropine, 1 hr after DFP	45·0 ± 10·4 (7)	< 0·005

Groups of 3 mice each were injected PAM or TMB-4 at 0·1 m-mole/kg either alone or admixed with atropine at 0·05 m-mole/kg. The animals were decapitated after 30 min and the blood was analysed for the oximes according to Sundwall.[20] DFP was injected at 3·4 mg/kg in some cases before the oximes or oxime-atropine mixture. Readings were taken at 335 mμ for PAM and 345 mμ for TMB-4. Control blood samples gave values equivalent to 30·5 ± 7·5 mμmole of PAM and 12·0 ± 3·5 mμmole of TMB-4 (6 trials each) for which corrections have been made in the Table above.

interest that atropine itself increases the blood concentration of TMB-4, which is further enhanced in DFP-treated animals. All the differences are statistically significant ($P < 0{\cdot}005$).

In the case of PAM the differences are less significant though the trend is perceptible. The PAM concentration in animals treated first with DFP and then with PAM and atropine is significantly higher than that in animals treated only with PAM and atropine ($P < 0{\cdot}001$). A disadvantage in the method is the high values for blanks (equivalent to $30{\cdot}5 \pm 7{\cdot}5$ mμmole of PAM/ml of blood) which may mask the differences if any in the actual values.

DISCUSSION

DFP and eserine induce a condition in mice in which $DI^{32}P$, which is itself physiologically inert and acts as a tracer compound, accumulates in the tissues and blood (Table 1). $DI^{32}P$ is not actively transported in the cell as the S/M ratio is never more than 0·7, but it probably enters the cell by free diffusion. The accumulation of $DI^{32}P$ is not in any way linked to the transport ATPase system, which even under the near-lethal DFP dosage employed remains unaffected in the kidney, brain and liver (Table 2).

The accumulation of $DI^{32}P$ may be due to the well-known effects of anti-cholinesterases on the cardio-vascular system. These are far too complicated and may be due to central, reflex and peripheral actions.[22] The most perceptible effect (in all species studied except the rat)[23] is a depression of blood pressure and a slowing of the heart rate. This condition may result in a decreased glomerular filtration rate of $DI^{32}P$ and thus its accumulation in blood. During the longer period of stay in blood it probably diffuses to a larger extent into the liver and other cells.

Atropine seems to correct this condition. Toxogonin has a slight influence (Table 3). TMB-4 has the least effect. Milošević[24] found that the paraoxon or physostigmine-induced blood-pressure rise in rats could be reduced by Toxogonin but not by PAM.

The practical significance of these results is that in an animal with a depressed circulatory rate the oximes may be at a higher concentration in blood than in normal animals but that they may not be able to circulate sufficiently rapidly to effect reactivation of the phosphorylated AChE. It is also probably vital that the blood containing the oximes should be pumped at sufficient pressure to reach vital tissues such as the brain.

It is a common experience in toxicity studies of organophosphorus esters that most of the deaths occur in the initial few hours. Those which survive this period continue to live indefinitely. Atropine if injected prophylactically prolongs life for a few more hours[2] but the animals ultimately die from doses not much above the normal LD_{50}.

The potentiating effect of atropine on the action of oximes may thus be due to (1) giving protection to animals in the initial critical hours, (2) restoring the circulatory rate to normal, thereby effecting a higher turn-over of oximes and at a greater pressure, and (3) maintaining a higher concentration of oximes in blood (Table 4). Mayer and Bain[4] have emphasized the importance of the concentration gradient between the blood and the brain in study of penetration of quaternary compounds across the blood-brain barrier.

Firemark *et al.*[8] observed a higher concentration of ^{14}C-PAM in certain parts of the

brain of rats which had been treated with Dipterex. They suggested that this was due to the inhibition of an active transport system which transported PAM out of the brain *in vivo*. The results obtained in this paper may partially explain their findings as being due to anti-cholinesterase-induced higher concentration of PAM in blood which because of the increased concentration gradient penetrates more into the brain tissue.[4]

The effect of atropine itself in maintaining a higher concentration of TMB-4 in blood (and also perhaps of PAM) (Table 4) is not understood. No information is available as to how TMB-4 is excreted, though PAM is known to be partly excreted by active transport.[21] Atropine probably interferes with this mechanism. The practical significance of atropine preventing the dissipation of oximes is obvious.

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