Biochemical Pharmacology, 1966, Vol. 15, pp. 1577-1587. Pergamon Press, Ltd., Printed in Great Britain.

DISTRIBUTION OF DF^{32}P IN MOUSE ORGANS—II

THE EFFECT OF PROPHYLACTICALLY ADMINISTERED OXIMES AND ATROPINE ON INCORPORATION AND TOXICITY

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(*Received* 28 *February* 1966; *accepted* 30 *March* 1966)

Abstract—A mixture of diisopropyl phosphorofluoridate (DFP) and radioactive diisopropyl phosphorofluoridate (DF^{32}P) was injected at various dose levels to mice which had been pre-treated with certain pyridinium aldoxime antidotes and atropine. The total as well as the trichloracetic acid (TCA)-soluble radioactivity incorporated in the liver, kidney, lung and brain was determined, from which the bound DFP-derived phosphorus was calculated. Bound ^{32}P in the organs attains a certain level rapidly even at 1 or 2 LD_{50}s of DFP, after which it does not perceptibly increase on raising the dosage. The presence of prophylactically-administered oximes and atropine does not seem to influence the amount of DFP which is bound to the tissue. The acid-soluble DFP, on the other hand, increases in the organs roughly proportional to the log of the dose administered. There is no correlation between the bound ^{32}P in any organ and the onset of death. In the liver, the bound ^{32}P which is approximately constant at all DFP levels, is mainly in the microsomal fraction, while the acid-soluble part, which varies with the dose, is localized in the supernatant. The latter is detoxicated and eliminated more rapidly than the former. As a preliminary to these studies, the LD_{50} of DFP to mice against various antidotes was determined under standard conditions.

CERTAIN oximes of the pyridinium type[1-5] are powerful reactivators of acetylcholinesterase (AChE) which has been inhibited irreversibly by organophosphorus toxic esters. In conjunction with atropine they are also effective antidotes in being able to protect experimental animals against several doses of organophosphates.[6, 7] Many cases of accidental insecticide poisoning in man have been treated successfully by administering oxime antidotes.[8]

Whereas the mode of action of oximes as reactivators is fairly clear as being due to their ability to displace the phosphorus-containing moiety from the inhibited AChE,[2, 9] their effect as antidotes is not wholly ascribable to this mechanism.[10] There are several complicating factors which have been discussed in detail[6, 11] but some of them may be briefly mentioned.

At marginal doses the AChE levels of dead and surviving animals do not show any differences.[12] There are wide variations in the AChE contents of different organs at the time of death, and even for a particular organ, these are not the same when different organophosphates are used.[13] Thus, the extent of contribution of the oxime-induced reactivation to the recovery of the animal is not correctly assessable.

It has been shown that the oximes themselves are poor antidotes even when administered prophylactically, but that they are able to protect an animal against the poison if given in combination with atropine, which by itself is also a weak antidote.[10] This

potentiating effect is not clearly understood. It is sought to be explained by the well-known anti-muscarinic and central effects of atropine which prolong life giving more time for the oxime to exert its effect. The oximes, due to their ionised nature, are unable to penetrate the blood-brain-barrier to an appreciable extent,[13–15] and their effect is, therefore, likely to be predominantly peripheral.

The oximes also directly react with organophosphates, reducing their half-life from several hours[16] to a few minutes,[17] but the contribution from this source to the over-all antidotal effect is said to be not appreciable.[7, 13] The oximes have pharmacological effects *per se*[18, 19] and it is difficult to determine how much these "other" actions contribute to the recovery of the animal.

The role of the oximes as causal therapeutic agents pre-supposes the initial phosphorylation of the vital enzyme, AChE, at the esteratic site by the organophosphate resulting in a stable covalent bond. The quaternary ammonium group of the oxime is then bound to the anionic centre or centres by coulombic forces and the molecule, thus oriented, makes a nucleophilic attack on the phosphoric ester group, resulting in its ultimate release from the enzyme.

By injecting an animal prophylactically with an oxime and atropine, however, it is possible to administer very large amounts of an organophosphate, a quantity which may be much more than the amount needed to phosphorylate all the esterases present in the animal. The results in the previous paper[20] indicated that the esterase content of the organs of the animal may be limited, being perhaps equivalent to only a few LD_{50}s. It is thus not clear how an animal can survive, even temporarily, a quantity of organophosphate which will be more than sufficient to inhibit and saturate all the esterases in its body.

In the present study an attempt has been made to determine how DFP is distributed in mouse organs when administered against various oximes and atropine. A mixture of DFP and $DF^{32}P$ has been used in these experiments. The total and the TCA-soluble radioactivity has been determined in the liver, the kidney, the lung and the brain to obtain an estimate of the approximate amount of the "bound", DFP-derived phosphorus which will be a measure of the extent of esterase inhibition.

The results indicate that the bound ^{32}P attains a certain equilibrium rapidly with or without antidotes, after which it does not show a measurable increase. The acid-soluble ^{32}P, on the other hand, increases rapidly and is also eliminated at a very fast rate. The protection afforded by oximes and atropine is thus not in preventing or reducing the incorporation of DFP in the organs. The bound P is mainly in the microsomal fraction, which has been shown previously[21, 22] to contain the major portion of the cell-esterases. The acid-soluble part is in the supernatant which contains most of the DFPase.[23]

The LD_{50}s of DFP for mice against oximes and atropine under comparable conditions are not available in literature. These have therefore been determined as a preliminary to these studies.

MATERIALS AND METHODS

In general, these were the same as reported in the previous paper.[20]

DFP was supplied by Sigma Chemical Co., St. Louis. 2-formyl-1-methyl pyridinium chloride oxime (2-PAM) and 1,1′-trimethylenebis (4-formyl pyridinium dichloride) dioxime (TMB-4) were obtained from the Research Institute of National Defence,

Dept. 1, Sundbyberg 4, Sweden. Bis-(4-hydroxyimino-methyl-pyridinium-1-methyl) ether dichloride (Toxogonin) was a product of Merck, Darmstadt, and 2,3-butanedione-2-oxime (DAM) of Hopkin and Williams. $DF^{32}P$ was obtained from the Radiochemical Centre, Amersham.

Stock solutions of DFP (10% w/w) and $DF^{32}P$ (10 μmole/g) were prepared in dry propyleneglycol and preserved at −16°. Quantities were weighed out and dissolved in physiological saline just before experiments. $DF^{32}P$ contained about 10–15 per cent of a radioactive impurity which was not extractable by ether. Since DFP hydrolyzes spontaneously in aqueous solutions, fresh batches were weighed and diluted every 1 hr when experiments were carried out over a prolonged period. DFP solutions above a concentration of 15 mg/ml (60 mg/kg body weight of mouse) were made by weighing the pure liquid and dissolving it in ethanol and then diluting with saline. The final solutions contained 10–20 per cent alcohol.

Albino mice of 20–25 g were used. In any series, animals of ±1 g were selected of a particular sex, mostly males. The injection volume of any substance was 4 ml/kg. The oximes were at 100 μmole and atropine at 50 μmole/kg in all the experiments. The antidotes were invariably administered 15 min before the DFP.

Toxicity determinations were carried out by injecting four sets of six mice each with DFP at dosages whose logs increased by a constant factor (*d*). A period of 24 hr was allowed for death or survival and the LD_{50} was calculated by the method of moving averages (Thompson)[24] using the tables compiled by Weil.[25]

For the study of incorporation of radioactivity in organs, groups of five or more mice were injected with antidotes intraperitoneally (i.p.) followed by DFP + $DF^{32}P$ 15 min thereafter subcutaneously (s.c.). The animals were sacrificed 30 min after the injection of the organophosphate and the organs were analyzed for radioactivity as described in the previous paper.[20] The brain tissue was also homogenized in saline instead of dissolving it in NaOH as previously. After taking aliquots for radioactivity determinations, the remainder of the homogenate was precipitated by an equal volume of 5% (w/v) TCA and aliquots of the filtrate were used for radioactivity determination after neutralization with alkali. Each animal was worked up separately. The values are expressed as DFP-derived P (mg × 10^5) in whole organs. Standard deviations are not included in the tables. The subcellular fractions were prepared by conventional methods. In the following text P refers to DFP-derived phosphorus.

RESULTS

LD_{50} *of* DFP *for mice against various antidotes*

The values available in literature for the LD_{50} of DFP to mice against various antidotes pertain to such widely different conditions of dosages of the oximes and atropine, intervals between antidotes and the DFP, and routes of administration, that comparisons are difficult. As a preliminary to the present studies, the LD_{50}s were determined employing certain standard conditions. The oximes were invariably at 100 μmole/kg. and atropine at 50 μmole/kg both administered in a volume of 4 ml/kg, 15 min before challenge by DFP. The observation period was 24 hr.

The results are given in Table 1. In general, the LD_{50} by the i.p. is greater than by the s.c. route as reported in the previous paper.[20] 2-PAM, DAM and atropine, administered alone do not raise the LD_{50} appreciably, but an observation of interest was that atropine keeps the animals alive for a longer period than the oximes. For

example, whereas death will be instantaneous at a dose of 8 mg/kg against 2-PAM and DAM, the animal can survive this dose for some hours if protected by atropine.

At equimolar doses the protection given by TMB-4 is the highest when used alone. The potentiating effect of atropine is greatest in the case of Toxogonin raising the LD_{50} from 15 mg to 159 mg/kg. It is lowest for DAM. The LD_{50} values for DFP against

TABLE 1. LD_{50} OF DFP FOR MICE AGAINST VARIOUS ANTIDOTES ADMINISTERED PROPHYLACTICALLY

No.	Antidote	*d*	s.c.		i.p.	
			LD_{50} (mg/kg)	Range, 95% confidence limits (mg/kg)	LD_{50} (mg/kg)	Range, 95% confidence limits (mg/kg)
1.	None	0·03	3·9	3·7–4·0	6·9	6·2–7·3
2.	Atropine	0·10	4·8	4·3–5·3	11·1	8·7–12·5
3.	DAM	0·10	4·3	4·0–4·6	9·3	7·6–11·2
4.	DAM + atropine	0·10	5·0	4·5–5·6	11·7	10·3–13·2
5.	2-PAM	0·10	4·8	4·3–5·4	11·4	10·1–12·8
6.	2-PAM + atropine	0·20	25	17–35	27	19–40
7	Toxogonin	0·20	15	12–20	22	19–25
8	Toxogonin + atropine	0·20	159	129–195	79	64–99
9.	TMB-4	0·20	43	35–60	54	42–69
10.	TMB-4 + atropine	0·20	135	73–251	86	74–100

LD_{50} was determined using four groups of six mice each. Atropine was at 50 μmole and other antidotes at 100 μmole/kg. The oximes and oxime–atropine mixtures were freshly prepared in physiological saline and injected in a volume of 4 ml/kg either i.p. or s.c. After exactly 15 min a solution of DFP was injected by an alternate route. The DFP solution was prepared by weighing out the exact amount of a 10% (w/w) stock solution in propylene glycol and dissolving it in the required volume of saline. For dose levels above 60 mg/kg, pure DFP was weighed and dissolved in ethanol before dilution with saline. The final alcohol content ranged from 10 to 20 per cent. The dosages of DFP were arranged in a geometric progression, the logarithms differing by a constant factor (*d*). A 24 hr observation period was allowed for death or survival, The LD_{50} was calculated according to Thompson[24] using the tables compiled by Weil.[25]

Toxogonin + atropine and TMB-4 + atropine can be taken to be only approximate since the DFP solutions were made from ethanolic solutions and contained 10 to 20% ethanol ultimately. Hobbiger[10] had observed that ethanol modified the toxicity to a certain extent, though the quantities involved in these studies (0·01–0·02 ml per mouse) are much less than those used by him. Moreover, at higher doses of DFP the s.c. absorption is probably slow and incomplete. There was invariably a swelling at the site of injection with the possibility of ejection of a small amount of the material. The slow absorption may also result in a higher detoxication by DFPase. It is seen that the LD_{50}s by the i.p. route in these cases are lower than those by the s.c. route, in contrast with all other values.

Distribution of DFP-*derived* P *in organs at* 30 *min*

These experiments were carried out according to a previously worked out time-schedule, five to seven animals being used for each assay. The antidote was administered i.p. followed by a mixture of DFP and $DF^{32}P$ s.c. 15 min thereafter. Sacrifice was at 30 min after DFP. The radioactivity injected to each mouse was kept constant in the whole series, subject to correction only for decay of ^{32}P. The dose of unlabelled

DFP was varied, the increase being arranged in logarithmic progression. The radioactivity incorporated in the organs was recalculated as DFP-derived P and expressed in mg as for whole organs (multiplied by the factor 10^5 to give convenient figures). Each animal was worked up separately and the averages are given in Table 2.

TABLE 2. DISTRIBUTION OF $DF^{32}P$ IN MOUSE ORGANS WHEN ADMINISTERED AFTER OXIME-ANTIDOTES AND ATROPINE

No.	Antidote	Dose DFP+ $DF^{32}P$ (mg/kg)	DFP-derived P (mg × 10^5)							
			Liver		Kidney		Lung		Brain	
			T	B	T	B	T	B	T	B
1	None	4	226	139	78	15	10	3·7	5·4	1·7
2	Atropine	4	217	146	45	13	7	3·0	4·2	1·5
3		8	453	203	102	5	15	4·3	8·0	2·1
4	2-PAM	4	212	128	53	12	10	3·3	5·1	1·4
5	2-PAM + atropine	4	277	194	45	14	7	2·8	4·5	1·6
6		8	414	218	99	16	14	4·0	9·0	2·9
7		16	659	241	214	30	20	5·7	15·5	2·5
8		24	1370	313	522	19	47	2·4	25·5	2·6
9	Toxogonin	4	256	153	47	12	7	2·7	4·3	1·2
10		8	457	197	142	7	19	5·2	10·6	2·4
11		16	747	229	336	45	36	10·4	24·3	2·2
12	Toxogonin + atropine	4	275	182	58	12	6	2·9	4·1	1·4
13		8	397	207	100	9	16	5·2	8·5	3·0
14		16	724	229	218	22	29	5·3	15·4	1·9
15		24	1045	254	360	22	35	5·5	20·4	1·3
16		32	1595	179	706	14	60	3·4	31·6	5·1
17		48	2098	236	1016	50	81	4·4	44·7	6·2
18		64	2562	385	1089	37	99	1·4	69·7	5·8
19	TMB-4	4	307	200	47	12	8	3·1	3·7	1·1
20		8	471	209	131	12	15	5·0	8·9	2·1
21		16	762	296	321	46	36	9·0	23·7	3·4
22		24	1254	299	511	33	60	3·5	31·0	4·3
23		32	1843	208	746	28	69	7·1	32·2	3·7
24		48	2327	179	998	49	79	12·5	62·6	4·0
25	TMB-4 + atropine	4	284	193	57	16	8	3·8	4·2	1·5
26		8	395	201	103	20	16	4·7	7·4	2·6
27		16	727	254	218	34	26	6·4	15·0	2·4
28		24	1078	265	443	3	48	4·5	24·9	2·7
29		32	1946	197	612	19	57	3·7	30·2	4·8
30		48	2510	318	971	31	76	4·4	44·7	3·5
31		64	2881	239	943	29	114	4·0	58·1	2·2

(T = total, B = bound). The values are expressed as for whole organs and represent the average of 5 to 7 animals. The conditions were: oximes (100 μmole/kg) and atropine (50 μmole/kg) were injected into mice i.p. in a volume of 4 ml/kg. After 15 min a mixture of DFP + $DF^{32}P$ in saline was administered s.c. and the animals were sacrificed 30 min after the DFP. The amount of radioactivity injected to each mouse in the series was constant while the dose of unlabelled DFP was varied. The organs were analyzed for total and TCA-soluble radioactivity and the bound ^{32}P was obtained by difference. The values are expressed as mg of DFP-derived P multiplied by the factor 10^5. Full details are given under Materials and Methods.

It is seen that the presence of an antidote does not seem to make a difference as far as the binding of DFP is concerned. At the level of 4 mg/kg which is the approximate LD_{50} of DFP for mice without antidotes, the amount which is bound is approximately the same in the controls as well as in the protected mice. The average percentage of bound-P to the total at this level under all conditions is 64·8 ± 3·8 (S.D.) for liver,

24·7 ± 5·0 for kidney, 41·4 ± 6·1 for lung and 32·2 ± 3·4 for brain. As the dose of DFP is increased, the total P increases in the organs but the bound-P seems to be stationary, or shows only minor variations. The divergences are much larger in the case of the kidney than for the liver and the lung. The bound-P in the brain shows a slight increase as the dose of DFP is raised. This may be due to the higher content of lipoid material in the brain which may take up the DFP in addition to the esterases.

A statistical treatment of the differences between the absolute values of bound-P in the organs under various conditions is not attempted as these figures have been obtained by difference, by subtracting the TCA-soluble from the total P, and are thus subject to large errors. These will be especially great at the higher dose levels of DFP when the bound-P represents only a few per cent of the total P. Nevertheless, the results indicate that, whereas the total P in the organ rises with the dose, the bound P remains at low levels.

The relationship between the dose and uptake is complicated at the 30-min period at which these experiments have been carried out. During this interval, a portion of the DFP is bound to the tissue, a certain portion is hydrolyzed by the detoxicating enzymes and some part is excreted rapidly. The uptake of radioactivity does not show linearity of relationship with the dose or the log-dose. If, however, the percentage of

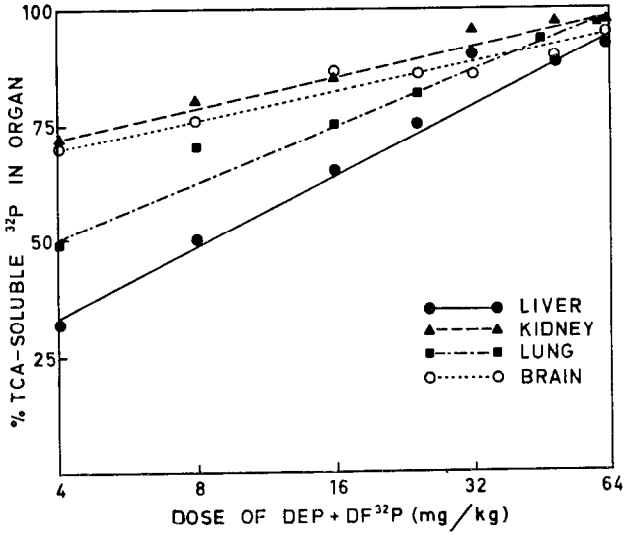

FIG. 1. The relationship between the DFP-derived P in mouse organs and the dose of DFP + DF^{32}P administered to mice against Toxogonin and atropine. The values and details pertain to those in Table 1. The abscissae are dose levels of DFP (in the logarithmic scale) and the ordinates represent the TCA-soluble DFP-derived P in the organs expressed as percentages of the total in the organs.

the acid-soluble P is plotted against the log-dose, approximate straight-line graphs are obtained. A set of graphs pertaining to DFP against Toxogonin + atropine is given in Fig. 1.

It has not been possible to establish a relationship between the amount of bound-P in any organ and the onset of death.

Distribution of DFP-*derived* P *in organs over a longer period*

Since it was found that the amount of bound-P in the organs of protected animals remained fairly constant even when the dose of DFP was increased, it was of interest to study the fate of the excess DFP over a longer period.

To a group of mice DFP + DF^{32}P was administered s.c. at 3·6 mg/kg at which they remained alive for a few days. To another set of mice which had been protected by Toxogonin and atropine, DFP + DF^{32}P was injected at 36 mg/kg. The amount of radioactivity injected was the same in both cases. Sets of five mice were killed at selected time intervals and the organs were analyzed for total and acid-soluble ^{32}P. The results are given in Table 3.

TABLE 3. DISTRIBUTION OF DF^{32}P DERIVED P IN MOUSE ORGANS OVER A LONGER PERIOD

Antidote	Interval between DFP and sacrifice (hr)	DFP derived P in whole organs (mg × 10^5)							
		Liver		Kidney		Lung		Brain	
		T	B	T	B	T	B	T	B
None	0·5	226	122	63	47	11·4	4·1	5·4	1·7
	6	155	128	20	12	5·5	4·1	2·6	1·9
	24	102	94	11	8	4·1	3·7	1·3	1·0
	48	81	78	7	6	2·5	2·1	2·1	1·8
Toxogonin + atropine	0·5	1242	193	429	5	62·1	11·5	31·6	5·1
	6	365	233	59	25	24·2	12·3	11·2	3·4
	24	177	149	28	18	9·2	6·6	6·2	1·9
	48	142	113	26	21	9·7	6·5	13·2	10·8
	96	123	99	14	13	5·7	4·2	10·3	10·0

DFP + DF^{32}P was at a level of 3·6 mg/kg for the control and 36 mg/kg against Toxogonin + atropine administered i.p. 15 min before the organophosphate. The animals were sacrificed at various intervals after the administration of DFP and the organs were analyzed as described under Materials and Methods. The values are expressed in mg × 10^5 as for whole organs and represent the average of 5 mice (T = total, B = bound).

It is observed that the total P in the organs is approximately five to seven times more in the Toxogonin-treated mice at the 30-min interval. This ratio rapidly falls to between 1 and 2 at the 24-hr interval in all the organs except the brain which shows a fall and then a rise.

Subcellular distribution of DFP

Our previous studies[21–23] with liver esterases have shown that DFP is predominantly bound to the microsomes. Since the present experiments indicate that only a fixed amount is bound to the tissues it was of interest to determine the subcellular distribution of the injected DFP. For this purpose, liver homogenates referred to in Table 3 were separated by differential centrifugation into the nuclear, mitochondrial, microsomal and supernatant fractions and the total and the TCA-soluble radioactivity in each fraction was determined. The results in Table 4 show that at the 30 min interval, in the untreated group, the major amount is present in the microsomes, while in the oxime-treated group most of the P is in the supernatant. It is also seen that the microsomal fraction consists almost entirely of bound-P while the supernatant fraction contains only negligible amounts of bound-P. There is a steep fall in the total P in the supernatant fraction of the treated group between 30 min and 6 hr.

These results confirm that the DFP injected in excess of the normal LD_{50} in animals protected with Toxogonin and atropine is in a loose form which is rapidly excreted.

Nature of DFP *in the Blood*

Two groups of mice were injected with DFP and $DF^{32}P$ as in the previous studies. One group had been protected with Toxogonin and atropine and received ten times the

TABLE 4. SUBCELLULAR DISTRIBUTION OF $DF^{32}P$-DERIVED P IN MOUSE LIVER

Antidote	Interval between DFP and sacrifice	DFP-derived P in subcellular fraction (mg × 10^5)							
		Nucleus		Mitochondria		Microsome		Supernatant	
	(hr)	T	B	T	B	T	B	T	B
None	0·5	34	22	26	20	85	82	77	4
	6	30	25	18	15	62	61	24	9
	24	21	20	15	14	46	46	12	6
	48	25	24	11	11	37	37	5	3
Toxogonin + atropine	0·5	174	44	105	36	140	115	785	59
	6	92	67	59	44	109	108	112	12
	24	43	37	24	24	66	64	24	3
	48	25	20	36	29	74	73	19	6
	96	24	21	14	13	31	28	14	3

The subcellular fractions were prepared from the livers mentioned in Table 3. The values refer to one mouse liver (T = total, B = bound).

TABLE 5. RATE OF HYDROLYSIS OF DFP IN THE MOUSE CIRCULATORY SYSTEM

Interval between DFP + $DF^{32}P$ and sacrifice (min)	Percentage of radioactivity not extractable by ether	
	Control group	Toxogonin-treated group
15	92·4	90·3
30	99·8	92·1
60	100·0	95·4
120	100·0	98·2

Two groups of mice were injected respectively with DFP + $DF^{32}P$ at 3·6 and 36 mg/kg, the amount of radioactivity injected being constant in both the groups. The latter group had been protected with Toxogonin and atropine. At various intervals two animals from each group were killed by decapitation and the blood was collected in heparinized cups. One ml portions were quickly diluted to 25 ml with cold saline and radioactivity was determined in aliquots both before and after ether extraction (3×). The values are given as percentages of the non-extractable (hydrolyzed DFP) to the total.

DFP administered to the control. Blood was collected by decapitation from sets of two mice each from each group into heparinized cups and rapidly analyzed for total radioactivity and radioactivity after ether extraction which would remove the free DFP. Results in Table 5 indicate that even at 15 min the injected DFP is mostly, but

not completely, decomposed. The degradation seems to be complete at 2 hr. It has not been determined whether the slight difference in the rates of decomposition of DFP between the two groups is statistically significant.

DISCUSSION

The mechanism by which oximes protect experimental animals against the toxic effects of organophosphorus esters is complex. It is the net result of at least 3 effects: (1) Reactivation of phosphorylated AChE; (2) the direct chemical reaction between the oxime and the organophosphate and (3) certain pharmacological actions which the oximes exert directly on the tissues other than by reactivation of AChE. It is considered that effect (1) is predominant (ref. 6, p. 960), the other two being subsidiary and contributive.

The direct chemical reaction between oximes and DFP cannot completely account for the high protecting effect of the oximes. The exact reaction between the oximes and DFP is not known, but from analogous studies with Sarin it can be assumed that the oximes themselves are decomposed in the reaction.[16, 17, 32, 33] Results in Table 1 indicate that a dose of 100 μmole/kg of the oximes is able to protect mice against several LD_{50}s of DFP. Even if the whole amount of the injected oxime takes part in the chemical reaction, not more than 18·4 mg/kg (100 μmole) of DFP in the case of 2-PAM and 37 mg/kg (200 μmole) in the case of the dioximes can be decomposed. On the other hand, doses of DFP very much above these levels are "safe" to mice in the presence of the respective oximes and atropine.

That the chemical destruction of DFP cannot contribute appreciably is also confirmed from the results in Table 2. The amount of $DF^{32}P$ which is bound to the organs is uniform and is not influenced by the presence of prophylactically administered oximes. If chemical degradation of DFP takes place to a large extent there will be less of it available for binding to the tissues esterases, and this will be reflected in the amount of bound-^{32}P in the organs.

It has not been possible to establish any relationship between the bound^{32}P in an organ (a measure of the enzyme-inhibition) and the occurrence of death. The injected DFP is found to bind itself to the tissues rapidly between 4 and 8 mg/kg and this process seems to be uninfluenced by the presence of prophylactically administered oximes (Table 2). After the bound-P reaches a certain level, which is apparently the saturation point of all esterases including AChE, as evidenced by the fairly constant values for bound-P, any excess DFP injected is found to be in a loose, acid-soluble form in the organs, increasing approximately according to the log-dose of DFP administered (Fig. 1).

It is at this stage that the sequence of events is not clear. DFP is known to penetrate all types of tissues freely and it is difficult to visualize a situation, however transient, in which the animal continues to live even after a complete inhibition of all the AChE in its system, which can be assumed to happen in the presence of such high amounts of DFP employed. (In fact, doses as much as 128 mg/kg have been tried in these studies but the results have not been included in Table 2 as the presence of enormous amounts of acid-soluble P vitiated the values for the bound-P). The time factor is against the concept of phosphorylation of AChE and its subsequent reactivation in these initial stages. Many authors have expressed doubts about reactivation of AChE being the sole mechanism of oxime-induced antidotal action.[10, 12, 27–29] The

antidotes seem to shield or protect the AChE or other vital receptors in some way reminiscent of the action of Ambenonium chloride (WIN 8077).[26] One may, however, speculate on the following lines. The functional AChE is external and is present on the cell membranes.[27, 34] DFP is freely permeable to the membrane but the quaternary oximes may not be so, atropine probably further influencing the low permeability.[35] Thus, there will be much more oxime available externally to protect or reactivate the functional AChE while rapid enzymic degradation of DFP is taking place within the cell.

The results in Table 3 show that in none of the four organs studied could a reduction be observed in the bound-P over a longer period as a result of the prior administration of antidotes. The DFP-inactivated enzymes change from the reactivable to the non-reactivable form quickly[10] and oxime-induced differences, if any, in the bound-P should have become apparent during the observation period of 96 hr up towhich the studies were conducted. In these experiments 2 groups of mice were injected respectively 3·6 and 36 mg/kg DFP, the latter against Toxogonin and atropine. The dose of 3·6 mg (without antidotes) is very close to the LD_{50} and there were in fact, some deaths. The dose of 36 mg/kg against Toxogonin and atropine represents only about a fourth of the LD_{50} under these conditions and the animals did not show any visible signs of intense toxicity. None of the animals died. If the toxic effects are due to the inhibition of the esterases, the extent of which is determined in this case by the amount of bound-P in the organs, this should be higher in the control group and lower in the Toxogonin-protected group. Actually the bound-P is much higher in the treated group in all the organs and this persists throughout the period of observation.

It is obvious that the capacity of the cell to bind DFP is limited, being equivalent to its esterase content. It has already been shown that the DFP-binding esterases are localized in the microsomes.[21–23] Thus, the DFP which is bound to the microsomes should be a fixed amount irrespective of the dose of DFP injected to the animal. The excess, un-combined DFP, should be found in some other fraction. This is confirmed in the results presented in Table 4. The microsomal P (almost the whole of which is bound) is approximately of the same magnitude in both the control and the treated group, in spite of the latter having received nine times more DFP than the former. The excess DFP in the Toxogonin-protected group is localized in the supernatant fraction, most of it in an un-combined form. While the rate of elimination of ^{32}P from the microsomes is slow and approximately of the same order in both the groups, the excretion of ^{32}P from the supernatant in the Toxogonin-treated group is extremely rapid, the major portion being lost between 0·5 and 6 hr. Thus the higher elimination of organophosphate in this group is not as a result of any reactivating process, since this DFP has not taken part in enzyme phosphorylation but has been hydrolyzed by the natural detoxicating mechanisms in the animal. It has already been shown that DFPase is localized in the supernatant fraction.[23] From the results in Table 5 it is evident that these processes are rapid.

The uptake of DFP in the brain is somewhat more complicated than in the liver and other organs, the "bound" P in this case showing an increase after a time. Such an observation has been recorded for Tabun also.[30] This may be because of the high content of lipids in the brain which may incorporate the organophosphate.

If the intracellular distribution of DFP in the brain cells is analogous to that in the liver cells, it is likely that small amounts of the oximes will be sufficient to protect the

vital centres from the lethal effects of DFP. It is known that oximes penetrate the blood-brain barrier to a limited extent.[15, 31]

Acknowledgements—The author is grateful to Prof. Gunnar Ågren for his interest, encouragement and many helpful suggestions. This work was supported by generous grants from the Swedish Medical Research Council (Project Nos. 40P-666-01 and 40X-665-01). Miss Annsofi Holst and Miss Margaretha Lindström rendered skilful technical assistance.

REFERENCES

1. A. F. Childs, D. R. Davies, A. L. Green and J. P. Rutland, *Br. J. Pharmac.* **10**, 462 (1955).
2. I. B. Wilson and S. Ginsburg, *Biochim. Biophys. Acta* **18**, 168 (1955).
3. F. Hobbiger, D. G. O'Sullivan and P. W. Sadler, *Nature, Lond.* **182**, 1498 (1958).
4. E. J. Poziomek, B. E. Hackley, Jr., and G. M. Steinberg, *J. org. Chem.* **23**, 714 (1958).
5. A. Lützinghaus and I. Hagedorn, *Arzneimittel-Forsch.* **14**, 1 (1964).
6. F. Hobbiger in *Handbuch der Experimentellen Pharmakologie, Ergänzungswerk*, Band XV, p. 921. Springer-Verlag, Berlin (1963).
7. H. Kewitz, I. B. Wilson and D. Nachmansohn, *Archs Biochem.* **64**, 456 (1956).
8. T. Namba and K. Hiraki, *J. Am. med. Ass.* **166**, 1834 (1958).
9. I. B. Wilson, *Fedn. Proc.* **18**, 752 (1959).
10. F. Hobbiger, *Br. J. Pharmac.* **12**, 438 (1957).
11. R. D. O'Brien, *Toxic Phosphorus Esters*, p. 175. Academic Press, New York (1960).
12. E. Edery and G. Schatzberg-Porath, *Science* **128**, 1137 (1958).
13. H. Kewitz and D. Nachmansohn, *Archs Biochem.* **66**, 263, 271 (1957).
14. T. A. Loomis, *Toxic. appl. Pharmac.* **5**, 489 (1963).
15. H. Firemark, C. F. Barlow and L. J. Roth, *J. Pharmac. exp. Ther.* **145**, 252 (1964).
16. B. E. Hackley, Jr., R. Plapinger, M. Stolberg and T. Wagner-Jauregg, *J. Am. chem. Soc* **77**, 3651 (1955).
17. B. E. Hackley, Jr., G. M. Steinberg and J. C. Lamb, *Archs Biochem.* **80**, 211 (1959).
18. R. Holmes and E. L. Robins, *Br. J. Pharmac.* **10**, 490 (1955).
19. K. Bethe, W. D. Erdman, L. Lendle and G. Schmidt, *Arch. exp. Path. Pharmak.* **231**, 3 (1957).
20. B. V. Ramachandran, *Biochem. Pharmac* **15**, 169 (1966).
21. B. V. Ramachandran, L. Engström and G. Ågren, *Biochem. Pharmac.* **12**, 167 (1963).
22. B. V. Ramachandran and G. Ågren, *Biochem. Pharmac.* **12**, 981 (1963).
23. B. V. Ramachandran and G. Ågren, *Biochem. Pharmac.* **13**, 849 (1964).
24. W. R. Thompson, *Bact. Rev.* **11**, 115 (1947).
25. C. S. Weil, *Biometrics*, **8**, 249 (1952).
26. G. B. Koelle, *J. Pharmac. exp. Ther.* **120**, 488 (1957).
27. J. H. Fleisher, J. Hansa, P. J. Killos and C. S. Harrison, *J. Pharmac. exp. Ther.* **130**, 461 (1960).
28. T. A. Loomis, M. J. Welsh, Jr., and G. T. Miller, *Toxic. appl. Pharmac.* **5**, 588 (1963).
29. I. W. Coleman, P. E. Little and G. A. Grant, *Can. J. Biochem. Physiol.* **38**, 1035 (1960).
30. E. Heilbronn, I.-E. Appelgren and A. Sundwall, *Biochem. Pharmac.* **13**, 1189 (1964).
31. W. D. Erdman, *Arzneimittel-Forsch.* **15**, 135 (1965).
32. A. L. Green and B. Saville, *J. chem. Soc.* p. 3887 (1956).
33. J. C. Lamb, C. M. Steinberg and B. E. Hackley, Jr., *Biochim. biophys. Acta* **89**, 174 (1964).
34. G. B. Koelle in *Handbuch der Experimentellen Pharmakologie, Ergänzungswerk, Band XV*, p. 224. Springer-Verlag, Berlin (1963).
35. B. V. Ramachandran and G. Ågren, *Acta pharmac. tox.* **20**, 339 (1963).