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EFFECTS OF SOMAN AND DFP *IN VIVO* AND *IN VITRO* ON CEREBRAL METABOLISM IN THE RAT

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Abstract—The effects of lethal doses of Soman and DFP *in vivo* and at a range of concentrations of these poisons *in vitro* on tissue respiration and on the activities of cholinesterase, succinate dehydrogenase, aldolase, hexokinase and ATPase in rat cerebral cortex have been studied. *In vivo*, lethal doses of Soman (0·16 mg/kg s.c.) and of DFP (3·2 mg/kg s.c.) significantly inhibited the uptake of oxygen by slices of rat cerebral cortex and also caused a significant depression of the activities of succinate dehydrogenase (33–43 per cent) and aldolase (17–36 per cent) and also an apparent decrease (26–43 per cent) in Na^+, K^+-ATPase. Hexokinase activity was unaffected, while cholinesterase activity was almost completely inhibited. Qualitatively similar effects were observed *in vitro*. It is concluded that a number of the metabolic effects of these organophosphate poisons are not related to their anticholinesterase activity.

MANY organophosphorus compounds are potent inhibitors of esterases, in particular of cholinesterase, producing a stable, inactive phosphorylated enzyme.[1] There is, however, evidence to suggest that some of these poisons have other direct effects unrelated to their anticholinesterase activity. This includes the irreversible and specific inhibition of Na^+, K^+-ATPase by DFP and other alkylating agents *in vitro*;[2,3] the delayed neurotoxic action of a number of these poisons *in vivo*;[4] the inhibition, *in vitro*, of many enzymes involved in carbohydrate metabolism by DFP,[5] and the inhibition by Soman,[6] DFP[7] and phosphamidon[8] of the oxygen uptake in slices of cerebral cortex of animals poisoned with lethal doses of these compounds. However, much of this evidence can be regarded only as suggestive. In studies on the inhibition of enzymes *in vitro* by organophosphorus compounds,[2,3,5] the high concentrations used make it unwise to postulate the occurrence of such effects *in vivo*. In this paper, we have attempted to relate the inhibition of a number of enzymes *in vitro* by Soman and DFP with depressed activities of these enzymes found in animals poisoned *in vivo* with these compounds.

MATERIALS AND METHODS

Materials. Soman (pinacolyl methylphosphonofluoridate) of 96 per cent purity was kindly supplied by Professor M. Milošević, Institute of Pharmacology, Belgrade, and a pure sample of DFP (diisopropyl phosphorofluoridate) by Professor F. Hobbiger, Middlesex Hospital Medical School, London.

Treatment of animals. Wistar albino rats of both sexes were used in all experiments. Control animals were injected subcutaneously with 1 ml 0·9% (w/v) NaCl solution

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whereas experimental animals were injected with the required dose of the poison dissolved in 1 ml of physiological saline. Animals injected with lethal doses of these poisons were killed at a time when observation of the signs of poisoning indicated that death was imminent. This period was usually about 30 min after injection. Control animals and those injected with sub-lethal doses of the poisons were killed 30 min after the injection.

Measurement of tissue respiration. Rats were stunned by a blow to the back of the neck and were exsanguinated by cutting across the carotid arteries. The brain was removed and two slices (about 0·35 mm thick) were cut from each hemisphere.[9] Each slice was weighed (30–50 mg) and immediately placed into Warburg vessels containing 3·2 ml of a medium of the following composition: NaCl 135 mM, KCl 5 mM, $MgSO_4$ 1·3 mM, $CaCl_2$ 0·5 mM, glucose 12 mM and phosphate buffer, pH 7·4, 0·01 M. The medium was equilibrated before use with Carbogen (95% O_2 + 5% CO_2). Respiratory rates were measured by the conventional Warburg technique[9,10] at 37°. Vessels were gassed for 5 min and then equilibrated for 10 min at 37° before readings were taken. Readings were subsequently taken at 10 min intervals for 60 min. When potassium stimulated respiration was being measured, readings were taken at 10 min intervals to establish the unstimulated respiratory rate and then KCl (final concentration 50 mM) was tipped in from the side-arm. Readings were then taken for a further 50 min. Results have been expressed in terms of μmole O_2/g tissue/hr and statistical analysis was by the Student *t*-test.

In experiments in which the effects of the poisons were to be examined *in vitro*, the compound was added in 0·2 ml of isopropyl alcohol at the start of the experiment, an equal volume of the alcohol being added to control vessels.

Tissue dispersion and fractionation. The animals were stunned by a blow to the back of the neck, exsanguinated, the cerebral hemispheres taken and white matter was removed. The grey matter (about 0·9 g) was placed within 3 min of death in an ice-cold Potter homogenizer containing 10 vol. 0·32 M sucrose, previously adjusted to pH 7·4 with KOH. Homogenization involved 10 passages of the Teflon pestle, rotating at 2300 rev./min with intermediate periods of cooling in ice. The resulting homogenate was fractionated in a Spinco model L ultracentrifuge, No. 40 head, as follows. Values for *g* quoted were average values calculated to the mid-point of the tube. A mixed nuclear and mitochondrial pellet was obtained by centrifugation at 15,000 *g* for 20 min. The supernatant was recentrifuged under the same conditions and the second pellet was discarded. The first pellet (P_1) was resuspended in the original volume of sucrose. The supernatant was centrifuged at 80,000 *g* for 95 min to yield the microsomal pellet (P_2) and this final supernatant fraction (S). P_1 and P_2 were used to determine Na^+, K^+-ATPase and cholinesterase activities, and P_1 for the determination of succinate dehydrogenase activity. Fraction S was used in the examination of hexokinase and aldolase activities.

Protein contents were determined by the method of Lowry *et al.*,[11] with crystalline bovine plasma albumin as standard.

Enzyme determinations

Adenosine triphosphatase activity was estimated in the presence of 60 mM tris-HCl, pH 7·4; 30 mM KCl; 3 mM $MgCl_2$; tris-ATP, 3 mM and when present, 100 mM NaCl. Reaction mixtures (final volume 1 ml) were preincubated at 37° for 5 min before addi-

tion of the tissue sample, and then for a further 5 min. Reactions were stopped by the addition of 2 ml of 6% (w/v) trichloracetic acid at 0°. Inorganic phosphate was determined in the deproteinized samples[12] and results were expressed as μmoles of phosphate/mg of protein/hr.

Acetyl-cholinesterase was determined spectrophotometrically by the acetylthiocholine method.[13]

Succinate dehydrogenase was assayed according to the method described by Porteus and Clark.[14]

Aldolase was assayed spectrophotometrically as described by Nicholas and Bachelard.[15]

Hexokinase was assayed spectrophotometrically as described by Bachelard and Goldfarb.[16]

RESULTS

Effects of Soman and DFP in vivo *and* in vitro *on the respiration of isolated slices of rat cerebral cortex*

In initial experiments *in vitro* a range of concentrations of Soman and DFP were used in order to determine those concentrations giving 50 per cent inhibition of oxygen uptake. At each concentration of the poison, four determinations were made of the oxygen consumption. Results are shown graphically in Fig. 1. As can be seen, the respiratory rate was depressed to a significant extent ($P < 0{\cdot}01$) at concentrations of Soman between 10^{-4} M and 10^{-2} M, and at concentrations of DFP of between 5×10^{-3} M and 5×10^{-2} M. The concentrations at which the respiratory rate was inhibited by 50 per cent were approximately 5×10^{-4} M for Soman and 5×10^{-3} M for DFP.

Further experiments were carried out *in vivo*. Dose levels employed were 0·08 mg/kg, 0·16 mg/kg and 0·32 mg/kg (0·65, 1·3 and 2·6 × LD_{50} respectively) for Soman and

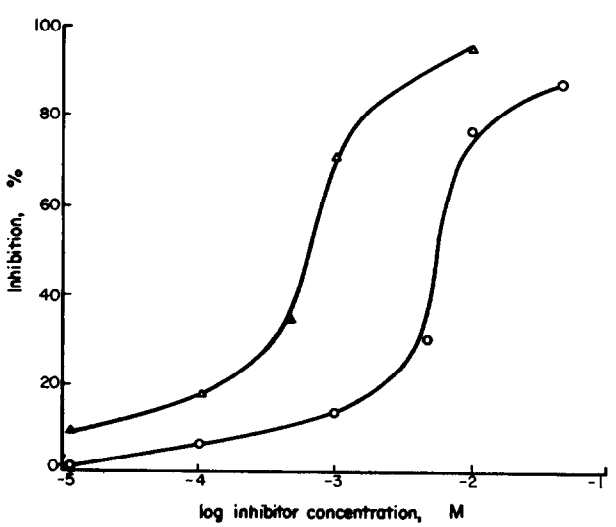

FIG. 1. Inhibition of oxygen uptake in slices of rat cerebral cortex by Soman and DFP. Oxygen uptake was measured manometrically in slices of rat cerebral cortex incubated with the indicated concentrations of Soman, △; and DFP, ○, as described in Methods.

3·2 mg/kg (1·3 × LD_{50}) for DFP. Tissue respiration in slices of cerebral cortex was measured in the absence and in the presence of added KCl as described in Methods. Results are presented in Table 1.

It is apparent that at all dose levels examined, the rates of oxygen consumption in unstimulated cortical slices obtained from the poisoned animals were depressed in comparison with those found in control preparations. The extent of the depression varied from 10 to 38 per cent of the control rate, depending on the dose administered, and was statistically significant in all cases ($P < 0·01$) except that in which a dose of 0·08 mg/kg of Soman was administered.

Table 1. Effects of Soman and DFP on tissue respiration in slices of rat cerebral cortex *in vivo* and *in vitro*

	Q_{O_2} μmole O_2/g of tissue/hr*		Inhibition (%)	
	(a) 5 mM KCl	(b) 55 mM KCl	a	b
Treatment	*In vivo*			
Saline (12)	110·2 ± 3·7	188 ± 4·2	—	—
Soman 0·08 mg/kg (5)	99·3 ± 2·8	185 ± 5·1	10	1·2
0·16 mg/kg (5)	79·7 ± 2·2†	162 ± 4·3†	27·7	15
0·32 mg/kg (5)	68·6 ± 3·1†	154 ± 3·8†	37·8	18·2
DFP 3·2 mg/kg (6)	82·2 ± 4·4†	160 ± 4·6†	25·5	15
Additions	*In vitro*			
Soman $5·5 \times 10^{-4}$ M (5)	61·4 ± 1·8†	123·4 ± 3·1†	45·3	34·5
DFP $5·5 \times 10^{-3}$ M (5)	72·6 ± 2·1†	151 ± 5·6†	34·2	19·7

* Oxygen uptake was measured manometrically in slices obtained as described in Methods. Respiratory rates obtained in slices from saline injected animals were used as the control for *in vitro* studies. Results are expressed as mean ± S.E.M.

Significantly different from control values: †$P < 0·01$.

As expected, potassium had a pronounced stimulatory effect on respiration in control preparations. This effect was also observed in preparations obtained from poisoned animals, though the rate of oxygen uptake was again depressed in comparison with the control. The extent of the depression was less than that found in the unstimulated case, ranging from 1·2 to 18 per cent of the control. Again, in all cases except that in which a dose of 0·08 mg/kg of Soman was administered, the depression of the respiratory rate was statistically significant.

When the poisons were added *in vitro* to preparations obtained from untreated animals, similar but much more marked effects were observed.

Enzyme inhibition in vivo *and* in vitro *by Soman and DFP*

Cerebral Na^+, K^+-ATPase activities were compared in the microsomal (P_2) fractions from animals poisoned with lethal doses of Soman or DFP with those of control animals. In some experiments the activity of the ATPase in preparations obtained

from control animals was determined after preincubation of the tissue preparations for 10 min at 25° with $5{\cdot}5 \times 10^{-4}$ M Soman or with $5{\cdot}5 \times 10^{-3}$ M DFP. Results are presented in Table 2.

The administration of lethal doses of Soman or DFP caused an apparent reduction in ATPase activity in the presence of sodium but not in its absence. The decrease in activity was consistent but, due to the wide variation of enzymic activity amongst different animals, was not statistically significant. *In vitro*, these poisons produce virtually complete inhibition of ATPase activity in the presence and in the absence of sodium. The latter findings are in agreement with those of other workers[2,3] who reported the inhibition of the Na^+, K^+-ATPase *in vitro* by a variety of acylating agents, including DFP.

TABLE 2. EFFECT OF SOMAN AND DFP ON RAT CEREBRAL ATPASE ACTIVITY *IN VIVO* AND *IN VITRO*

Fraction		ATPase activity* (μmoles P_i/mg of protein/hr) (a) Na^+ present	(b) Na^+ absent	a–b	Inhibition (%)
			In vivo		
Treatment					
Saline (5)	P_1	22·2 ± 2·3	10·2 ± 0·9	12·0	—
	P_2	22·8 ± 1·9	14·8 ± 1·2	8·0	—
Soman (4)	P_1	17·4 ± 1·6	10·6 ± 0·8	6·8	43·4
0·16 mg/kg	P_2	18·1 ± 1·3	12·3 ± 1·2	5·5	27·5
DFP (4)	P_1	18·3 ± 0·9	11·2 ± 1·1	7·1	41·0
3·2 mg/kg	P_2	19·7 ± 1·4	12·8 ± 1·3	5·9	26·3
			In vitro		
Additions					
Soman	P_1	0·55 ± 0·01†	0·35 ± 0·01†	0·20	97
$5{\cdot}5 \times 10^{-4}$ M	P_2	0·21 ± 0·01†	0·10 ± 0·005†	0·11	98
DFP	P_1	1·16 ± 0·08†	0·56 ± 0·02†	0·60	94
$5{\cdot}5 \times 10^{-3}$ M	P_2	0·66 ± 0·01†	0·25 ± 0·01†	0·41	96

* ATPase activity was assayed in nuclear and mitochondrial (P_1) and microsomal (P_2) fractions, as described in Methods. Results are expressed as mean ± S.E.M.
Statistically different from control values: †$P < 0{\cdot}001$.

Succinate dehydrogenase and aldolase activities were measured in the fractions prepared as described in Methods, obtained from control animals and from animals dosed with 0·16 mg/kg of Soman or with 3·2 mg/kg of DFP. Results are shown in Table 3. The effects of the poisons *in vitro* on succinate dehydrogenase activity were examined by determination of the reaction rate after preincubation of the tissue preparation at 25° for 10 min with $5{\cdot}5 \times 10^{-4}$ M Soman or with $5{\cdot}5 \times 10^{-3}$ M DFP. Aldolase activity at saturating substrate concentration (5 mM) was determined after preincubation of the tissue fraction at 25° for 10 min with concentrations of Soman ranging from 10^{-6} M to 10^{-2} M. Results are presented graphically in Fig. 2.

It is apparent that both succinate dehydrogenase and aldolase activities are depressed in preparations obtained from animals poisoned with lethal doses of Soman

Table 3. Effects of Soman and DFP on cerebral succinate dehydrogenase (SDH) and aldolase activities *in vivo* and *in vitro*

	SDH activity* (units [13]/mg of protein)	Inhibition (%)	Aldolase activity* (μmoles/mg protein/min)	Inhibition (%)
		In vivo		
Treatment				
Saline (5)	0·054 ± 0·002	—	0·069 ± 0·002	—
Soman (5) 0·16 mg/kg	0·031 ± 0·002†	42·3	0·043 ± 0·001†	36·8
DFP (5) 0·32 mg/kg	0·035 ± 0·003†	33·7	0·056 ± 0·002†	17·1
		In vitro		
Additions				
Soman (5) $5·5 \times 10^{-3}$ M	0·004 ± 0·0004†	91·6	0·010 ± 0·0004	85·4
DFP (5) $5·5 \times 10^{-3}$ M	0·005 ± 0·0006†	89·5	0·019 ± 0·002	72·5

* SDH activity was measured colorimetrically in the whole particulate fraction ($P_1 + P_2$), and aldolase activity was measured spectrophotometrically in the soluble supernatant (S) fraction as described in Methods. Preparations from saline-treated animals were used as controls for *in vitro* studies. Results are expressed as mean ± S.E.M.

Significantly different from control. †$P < 0·001$.

or DFP. *In vitro*, succinate dehydrogenase was strongly inhibited by these compounds, at the concentrations used. In striking contrast, hexokinase was not inhibited *in vitro* to a significant extent by Soman in concentrations as high as 10^{-2} M.

Under the experimental conditions employed, aldolase did not appear to be very sensitive to inhibition *in vitro* by Soman, a relatively high concentration at 10^{-3} M

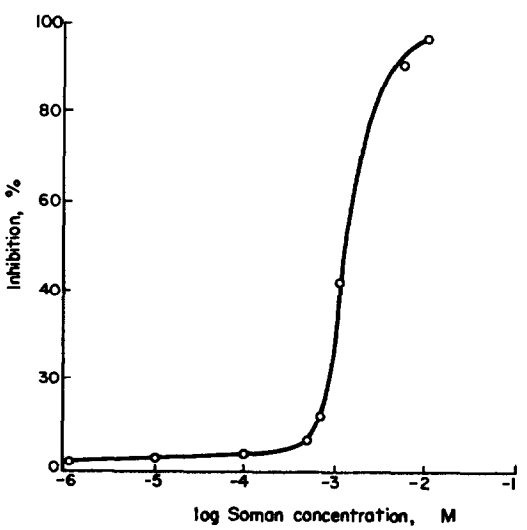

Fig. 2. Inhibition, *in vitro*, of aldolase activity by Soman. Aldolase activity was measured spectrophotometrically in the soluble supernatant (S) fraction, preincubated with the indicated concentration of Soman, as described in Methods.

having been required to produce 50 per cent inhibition. However, in kinetic experiments designed to characterize the nature of the inhibition, evidence was produced to suggest that aldolase is, in fact, extremely sensitive to inhibition by Soman, although in a manner that is not revealed by experiments of the above type. Aliquots of the supernatant fraction were incubated for 10 min at 25° with Soman at concentrations of 0, 10^{-6} M, 10^{-5} M and 10^{-4} M. The initial reaction velocities were then determined at different concentrations of the substrate, fructose-1,6-diphosphate. Plots of $1/v$ against $1/s$ are shown in Fig. 3. It will be seen that, in the range of concentrations studied, the degree and nature of the inhibition of aldolase by Soman is independent of the inhibitor concentration. The K_m of the inhibited enzyme was 14·5 μM compared with 2·5 μM for the uninhibited enzyme. A possible interpretation of these results is

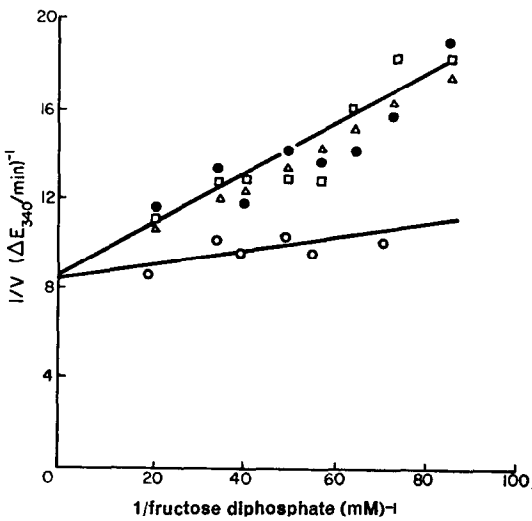

FIG. 3. Kinetics of Soman inhibition of aldolase. Reaction rates were measured in the presence of fructose-1,6-diphosphate in concentrations ranging from 12 μM to 50 μM using samples of the soluble supernatant fraction (S) that had been preincubated with Soman at concentrations of 0, ○; 10^{-6} M ●; 10^{-5}M, △; and 10^{-4}M, □, as described in Methods.

that a stable, phosphorylated enzyme was formed, having a much reduced affinity for the substrate, but having a maximum velocity equal to that of the untreated enzyme.

This finding is of particular interest insofar as it suggests that aldolase may be changed in its kinetic properties by those concentrations of Soman likely to be encountered *in vivo*. The inhibition observed *in vitro* at high concentrations of Soman in the presence of saturating substrate was probably of a different nature, as the maximum velocity was affected, and is probably of little significance *in vivo*, in view of the high concentrations required to produce this effect.

Cholinesterase activity. As expected, the injection of lethal doses of Soman (0·16 mg/kg) or DFP (3·2 mg/kg) *in vivo* resulted in almost total inhibition of cholinesterase activity in cerebral microsomal preparations obtained from the poisoned animals. In control animals cholinesterase activity was 6·1 $\pm$ 0·36 μmole/mg of protein/hr. In animals poisoned with Soman the activity was 0·056 $\pm$ 0·01 μmole/mg of protein/hr

(inhibition 99·4 per cent) and with DFP, 0·075 ± 0·02 μmole/mg of protein/hr (inhibition 98·8 per cent). The activity of this enzyme was estimated as a check on adequate penetration of the poisons to the brain. The actual level of inhibition *in vivo* is likely to be less than found here, due to release of the poisons from storage sites during homogenization; acetyl choline was not included in the homogenization medium to protect the enzyme.[17]

DISCUSSION

DFP and Soman produce all the symptoms expected in cases of organophosphate poisoning (convulsions, hypersalivation, bronchospasm, contraction of the diaphragm, acrocyanosis and muscular paralysis). There are two stages in the onset of these symptoms. The first is characterized by general excitation, and the second by paralysis and depression. It must be emphasised that in the experiments recorded here, when the animals were injected with these poisons, they were killed either 30 min later or, when lethal doses had been administered, late in the second phase of the poisoning. Experimental results may be critically dependent on the time of death. Paulet and André[18] reported that *in vivo*, organophosphates did not affect oxygen uptake by brain tissue. However, other workers have reported significant inhibition of respiration. Such observations have been made with respect to DFP,[7] Soman[6] and phosphamidon.[8] In the work reported in the latter two papers, animals were killed as in the present work. We have found (unpublished results) that no significant inhibition of oxygen uptake occurs in cerebral cortical preparations from animals killed during the first excitatory period of poisoning.

There appears to be two ways in which such inhibition may be brought about. The first is the result of the chain of events brought about by the accumulation of acetylcholine. This includes central respiratory inhibition; bronchospasm and associated hypersecretion in the respiratory tract; vasodilation and the consequent fall in blood pressure; neuromuscular paralysis of the diaphragm and intercostal muscles and violent convulsions of central origin, causing an increased oxygen demand upon an already overtaxed respiratory system.[19] In our own experiments (unpublished results) we have found that, after an initial period of hypertension during the excitatory phase of poisoning by Soman, circulatory failure and a fall in blood pressure develop during the depressive phase. In this context, the work of Schneider[20] is of interest. This author has shown that in experimental animals, if blood pressure is lowered to a critical level of 60–70 mm Hg, the autoregulation of cerebral circulation fails, leading to a marked reduction in blood flow. It was observed that such treatment could produce severe functional disorders, a reduction in oxygen uptake by the whole brain of up to 50 per cent, and the beginning of irreversible change to some cells. It would thus appear that the reduction of oxygen uptake observed in the present work might be explained solely in terms of the hypoxia and ischaemia developed as a result of the accumulation of acetylcholine.

However, some of our results are not compatible with this view and suggest that Soman and DFP may interfere with a number of metabolic processes independent of the esterases known to be inhibited by these compounds. Inhibition of the Na^+, K^+-ATPase was observed *in vitro* and possibly also *in vivo*. The inhibition *in vitro* is in agreement with that reported by Hokin *et al.*,[3] and by Sachs *et al.*[2] The latter authors proposed that the inhibition was specific and a result of the alkylation of a serine

residue at the active centre of the enzyme. It is possible that such a process could occur *in vivo*, but the high concentrations of the inhibitor required in the *in vitro* studies seem unlikely to occur *in vivo*.

Aldolase and succinate dehydrogenase are inhibited both *in vitro* and *in vivo*. It is unlikely that, in the case of succinate dehydrogenase, the effect *in vivo* is a result of hypoxia or ischaemia. Spector[21] reported that neither anoxia nor ischaemia alone appeared to produce irreversible changes in cerebral function in rats. Anoxic ischaemia did so and did lead to a reduction in cerebral succinate dehydrogenase activity, but only after a period exceeding 2 hr after such treatment. The observation in the present work that hexokinase was unaffected by Soman *in vitro* suggests that the inhibitions of aldolase and succinate dehydrogenase are relatively specific effects.

The mechanism of inhibition of aldolase by Soman is of considerable interest. At low concentrations of Soman, such as might be found *in vivo*, the effect of this compound is to produce a 6-fold decrease in the affinity of the enzyme for its substrate, having little effect on the maximum reaction velocity. Therefore, at low concentrations of Soman, little inhibition will be observed if the substrate is present in saturating concentration. It is possible that a number of other enzymes involved in carbohydrate metabolism may be affected in a similar fashion, and that the high concentrations of organophosphorus poisons reported to be required to inhibit the enzymes *in vitro* may reflect the fact that such studies have usually been made using only a single, sometimes saturating, concentration of substrate. If this is indeed the case, it is likely that carbohydrate metabolism is directly and profoundly disturbed in animals poisoned with these compounds and that this disturbance is a factor contributing to the depression of cerebral respiration observed in such animals.

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