Acta Neuropathol (Berl) (1983) 61:123–129

Acta Neuropathologica

Histological and Histochemical Changes in the Central Nervous System of the Rat Poisoned by an Irreversible Anticholinesterase Organophosphorus Compound*

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Summary. The effect of soman, a powerful organophosphorus (OP) cholinesterase inhibitor, was investigated in the central nervous system (CNS) of Wistar rats by neurohistology, histochemical mapping of acetylcholinesterase (AChE), and biochemical determination of cholinesterase (ChE) activity. Rats were poisoned by one lethal or sublethal subcutaneous (s.c.) injection or by several less strong weekly doses. When the acute cholinergic action of the OP led to severe respiratory failure and to repeated or prolonged convulsions, the surviving rats exhibited neuronal changes similar to those of hypoxic encephalopathy. In one case chronic intoxication gave rise to these symptoms and lesions after the fourth injection. The histochemical data showed that lesioned gray structures were generally poor in AChE. The enzymatic inhibition was quick and strong, but differed from one structure to another. ChE recovery was rapid until about 96 h after poisoning, the time course depending on the structure, but was incomplete even after 8 days. An attempt to correlate the initial level of ChE inhibition with the severity of the symptoms was not very conclusive. Our data suggest that the encephalopathy comes at least in part from complex hypoxic factors produced by the cholinergic crisis. The sequelae of slight hypoxic encephalopathy could account for some nervous long-term effects in men acutely poisoned by OP and surviving owing to mechanical ventilation.

Key words: Organophosphate intoxication – Rat – Neuropathology – Histochemistry – Acetylcholinesterase

Introduction

Most OPs are irreversible inhibitors of AChE. They hinder the degradation of acetylcholine released at central or peripheral cholinergic synapses. In sufficient concentration, they may give rise to a cholinergic crisis, leading to very severe acute intoxications, manifested in man by diverse muscarinic and nicotinic symptoms and by CNS effects: drowsiness, coma, convulsions (Koller and Klawans 1979). Acute OP poisoning may also cause intellectual and psychiatric sequelae, which may be transient or last several months (Namba et al. 1971; Sidell 1974). Only certain OPs are known to produce a delayed neuropathy (Abou-Donia 1981). Sustained exposure to low levels of OP may lead to electroencephalographic alterations, insomnia, neurobehavioral abnormalities, disorders of memory and concentration, and to psychiatric sequelae (Gershon and Shaw 1961; Dille and Smith 1964; Korsak and Sato 1977; Duffy et al. 1979). Necropsies in human deaths following OP poisoning are rare and show only non-specific changes in the CNS (Bidstrup 1950; Grob et al. 1950; Grob 1963; Davis et al. 1969).

Soman is a very powerful AChE inhibitor. It also has an "aging effect" on the enzyme, making it insensitive to reactivation by oximes, precious antidotes to OPs. The acute stage of poisoning or long-term exposure to such a highly toxic OP could provoke histological changes likely to explain the CNS sequelae observed in OP intoxicated men.

Therefore, in the present study, rats were poisoned with one LD_{50} or with several lower repeated injections of the toxic, and the histology of their brain was studied. In rats intoxicated by one LD_{50}, the topography of neuronal lesions was compared to the normal histochemical mapping of AChE. The time course and spread of AChE inhibition was studied using histochemistry and biochemical determination of the AChE activity. An attempt to correlate early AChE

* Supported by grants from the DRET (no. 79/1052 and no. 80/1103)
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inhibition with the severity of acute symptoms was made.

Materials and Methods

Animals

Wistar rats weighing 200 – 220 g were used.

Organophosphorus Compound

The soman (pinacolyl-methylphosphonofluoridate) was stored at – 40° C in dehydrated ethanol and diluted in saline before use.

Intoxication

Thirty-nine animals destined for histological examination were acutely intoxicated with a dose varying between 90 and 145 µg/kg. Eighteen rats were chronically intoxicated for the same purpose. They received at weekly intervals 2, 3, or 4 injections of 70 µg/kg of soman, the dose corresponding to the weight of the rats at the moment of the first injection. Animals destined for histochemistry and biochemical studies were injected with 100 µg/kg.

Histology

Acutely poisoned rats were killed 1 h, 6 h; 1, 2, or 4 days; 7 – 10 days; 16, 25, or 39 days after injection. Three chronically poisoned rats were killed at least 8 days following the 2nd, 3rd, or 4th injection. Three rats were also killed 2 or 3 weeks after the 4th injection. Before embedding in paraffin the CNSs were fixed either by immersion in 10% formaldehyde or by intracardial perfusion with the Susa-Heidenhain fixative according to Cammermeyer (1960). Coronal slices were taken from the following regions: telencephalon (two to four planes of section), mesencephalon (one plane), pons and cerebellum (one plane), medulla oblongata (one to two planes), spinal cord (one to three planes). Certain sections from paraffin-embedded blocks or frozen sections were stained with hemalun and phloxine and with the method of Woelcke for myelinated fibers. The atlas of Pellegrino et al. (1979) was used for easier localization of the CNS structures.

Histochemistry

The CNS was divided into three or four blocks which were frozen in isopentane cooled in liquid nitrogen. Frozen sections cut in a cryostat were treated according to Gomori's modification of the acetylthiocholine method (Gomori 1952) for AChE localization. Butyrylcholinesterase activity was inhibited by 10^{-6} M tetramonoisopropylpyrophosphortetramide (iso OMPA). For every histochemically stained section two adjacent sections were stained, one with cresyl violet for visualization of CNS gray matter and the other with hemalum and phloxine for histopathologic examination.

A coronal mapping of AChE staining of the CNS was performed in five control animals. The interval between two sections was 450 µm in the brain and about 200 µm in the brainstem and the cerebellum. In a preliminary experiment, one rat was killed 30 min, 2, 4, 7, or 8 days after poisoning for histochemical comparison with normal animals. The interval between two sections was 750 µm in the brain and 500 µm in the brainstem. In a further experiment, four coronal planes were chosen for the comparison: plane I passed through the striatum between the anterior and the middle third of the telencephalon; plane II passed through the interpeduncular nucleus (mesencephalon); plane III passed through the locus ceruleus (pons), and plane IV passed through the nucleus of the 12th nerve (medulla). The CNS of two or three poisoned rats was examined in each of the planes, 30 – 70 min, 24 h, 2, 4, 8, or 10 days after the injection.

Determination of ChE Activity

Owing to the relatively low level of CNS butyrylcholinesterases (Woolley 1963), the determination of the total ChE activity was considered to be a good approximation of the AChE level. Six to eight poisoned rats were sacrificed 90 min, 1, 2, 4, 8 and 15 days after poisoning. Six blocks of the CNS of each rat (cerebellum, medulla, mesencephalon, striatum, entorhinal cortex, neocortex) were homogenized in 10^{-2} M phosphate buffer at pH 7.4 containing 0.32 M sucrose and centrifuged at 1,800 rpm for 10 min. The resulting supernatants were reacted in a Technicon autoanalyser, according to the method of Ellman et al. (1961) modified by Voss (1968). The ChE measurements were arbitrarily expressed in absorbancy units per milligram of protein. Protein was measured by the method of Lowry et al. (1951) modified by Castaigne et al. (1965). Individual and mean values of the ChE inhibition in the different structures were expressed as a percentage of the mean enzymatic activity measured in six to 12 control rats.

Statistics

Biochemical results were compared using Student's *t*-test and variance analysis.

Results

Clinical Features

Single Poisoning. Injection of 145 µg/kg killed all the rats, whereas 100 µg/kg was close to the LD_{50}. At the early stage of poisoning, the rats presented a cholinergic crisis. The most visible muscarinic symptom was increased salivary secretion, and the most evident nicotinic symptoms were scattered fibrillations and fasciculations associated with muscle cramps. Involvement of the CNS was particularly demonstrated by drowsiness and generalized convulsions. The symptoms began within a few minutes after poisoning, resulted in respiratory failure and cyanosis and could last between a few hours and about 24 h. The convulsive seizures could recur for several days. Death generally happened within a few minutes or hours. During the 2 or 3 days following the acute stage, the rats were profoundly asthenic and hardly ate or drank. The general condition then improved. Clinical examination showed no neurologic sequelae after an experimental period of 39 days.

Repeated Poisoning. In one case death occurred 4 days after the first injection following slight initial symptoms. In two cases death happened a few minutes after the second and the third injection. Except for these cases, symptomatology was slight until the third injection. Among the ten rats given a 4th injection, one died 50 min later, and four showed severe acute symptoms lasting 24 h in one of the rats.

Histopathology

Single Poisoning. In animals killed 1 or 6 h after a single injection of 140, 120, or 100 µg/kg of soman, the

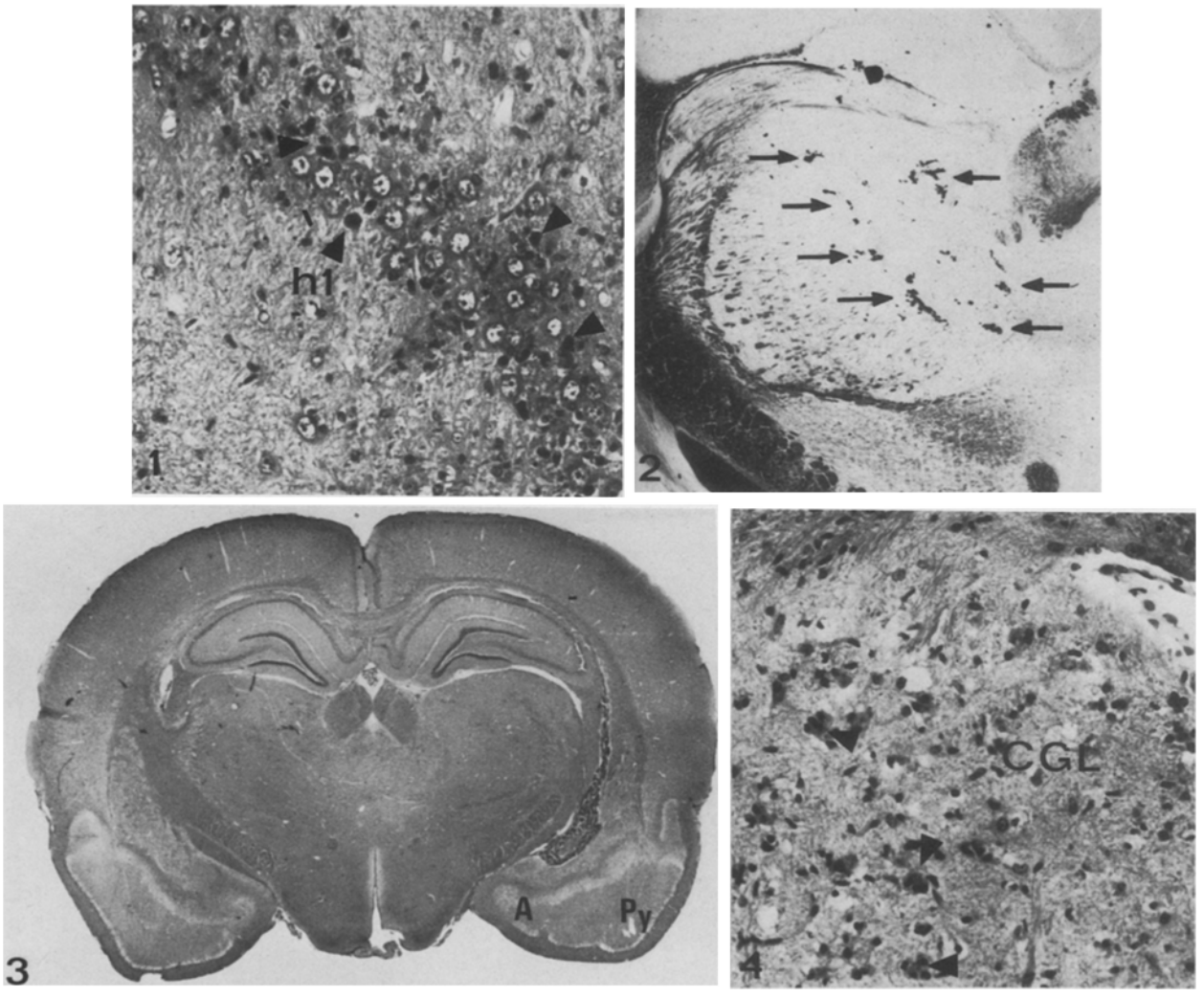


Fig. 1. Rat given four s.c. injections of 70 μg/kg of soman at weekly intervals and killed 8 days after the last injection. Perfusion fixation. Ischemic cell change (*arrowheads*) in the pyramidal layer of the hippocampus (h 1 sector). Hemalum and phloxin, × 240

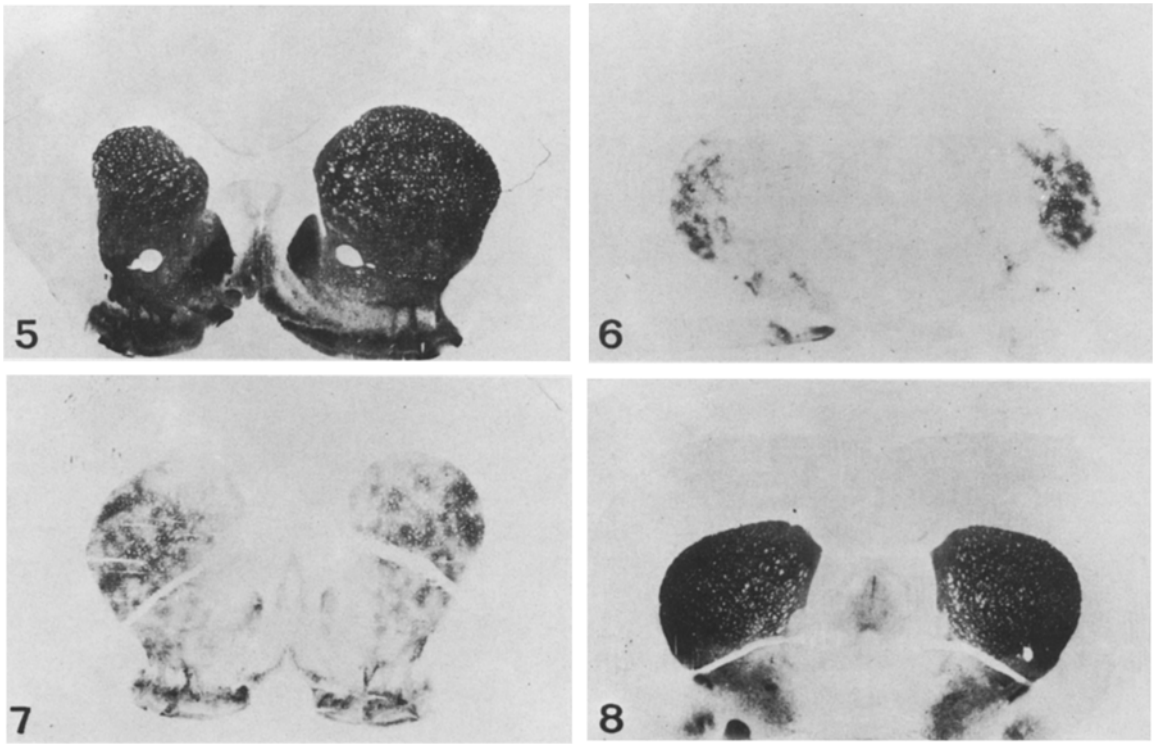
Fig. 2. Rat killed 24 h after one s.c. soman injection of 100 μg/kg. Fixation by immersion. Pericapillary hemorrhages in the thalamus (*arrows*). Woelcke, × 22

Fig. 3. Rat killed 24 h after a soman injection of 100 μg/kg. Fixation by immersion. Beginning of bilateral and symmetric ischemic necrosis of the pyriform cortex (*Py*) and of the amygdaloid complex (*A*). Hemalum and phloxin, × 7

Fig. 4. Corpus geniculatum laterale from the same rat as in Fig. 1. Several neurons are ghost cells surrounded with numerous glial cells (neuronophagia: *arrowheads*). Hemalum and phloxin, × 180

histological examination revealed only a more or less diffuse congestion, sometimes associated with small pericapillary hemorrhages.

Several rats killed 24 h after poisoning showed similar neuronal changes. A variable number of neurons of some structures of the gray matter exhibited eosinophilic cytoplasmic homogenization and pyknosis or nuclear lysis (ischemic cell change) (Fig. 1). The damaged structures, listed in order of frequency of injury, were the following (Table 1): the pyramidal layer of the hippocampus, the pyriform cortex, the neocortex, the claustrum, the entorhinal cortex, the nucleus lateralis septi, the thalamus, the gyrus dentatus, the amygdaloid complex, the cerebellard vermis, the striatum, the medial geniculate body, the inferior colliculus, the globus pallidus, the lateral geniculate body, the nucleus accumbens septi. Extensive neuronal changes of the thalamus were usually associated with thalamic capillary hemorrhages (Fig. 2). Extensive neuronal changes of the pyriform cortex and of the amygdaloid complex were often worsened by a spongy state of the corresponding neuropil (Fig. 3), similar to the onset of ischemic necrosis. Ten days and 16 days after poisoning, the destroyed neurons appeared as



Figs. 5–8. AChE staining of the rat brain by the method of Gomori before and after one s.c. soman injection of 100 µg/kg. Coronal plane of section though the striatum, × 7. **Fig. 5:** normal rat. **Fig. 6:** rat killed 24 h after poisoning. **Fig. 7:** rat killed 48 h after poisoning. **Fig. 8:** rat killed 4 days after poisoning

Table 1. Selective localization of neuronal damage observed in 13 soman poisoned rats

CNS structures	Number of affected rats
Hippocampus (pyramidal layer)	13
Piriform cortex	12
Neocortex	12
Claustrum	12
Entorhinal cortex	10
N. lateralis septi	10
Thalamus	8
Gyrus dentatus	7
Amygdaloid complex	6
Cerebellum (Vermis)	5
Striatum	4
Corpus geniculatum mediale	4
Inferior colliculus	4
Globus pallidus	3
Corpus geniculatum laterale	3
N. accumbens septi	3

ghost cells surrounded by glial nuclei (neuronophagia) (Fig. 4).

The occurrence of these neuronal lesions did not seem to correlate with the dose of injected poison. In fact, the lesions happened in only one of the four animals poisoned with 120 µg/kg, whereas they were present in nine of the 13 rats given 100 µg/kg. Moreover, the neuronal alteration, which was observed in 13 of the 39 poisoned animals, was only present in rats with severe and prolonged symptoms (respiratory distress, frequent, and persistent convulsions).

Chronic Poisoning. Only one animal, killed 8 days after the 4th injection, showed the neuronal lesions observed acute poisoning. It was the one which had presented severe respiratory distress and convulsive seizures.

Histochemistry

The histochemical data obtained from the CNS sections of poisoned rats showed that the AChE inhibition by a same dose of soman could vary from one animal to another. Individual variations were also observed in the recovery of the enzyme staining.

The general time course of reappearance of AChE staining after soman poisoning was as follows: After 30 min and 75 min most of the structures stained in normal animals (Fig. 5) were totally devoid of AChE activity. The staining was very slight in a few structures normally heavily stained: the striatum (neuropil), the pallidum (a few neurons), and some basal olfactory structures (the lateral nucleus of amygdala and the tuberculum olfactorium) in plane I, the nucleus in-

terpeduncularis in plane II, the nucleus of the VIIth nerve, the nucleus of the Vth nerve and the locus ceruleus in plane III, and the nucleus of the XIIthe nerve in plane IV. After 24 h, the AChE reappeared particularly in some perikarya of neurons from the above mentioned structures and to a lower degree elsewhere (Fig. 6). After 48 h (Fig. 7) and after 4 days (Fig. 8), the AChE staining was visible in most of the neuronal perikarya stained in normal animals. From about the 4th day after poisoning, the staining became generalized in the nerve fibers from AChE containing neurons. After 8 days, AChE was visible in every structure which normally contains AChE. The staining was, however, less intense than in normal animals. The time of recovery of nearly normal AChE staining varied from one structure to another according to the level of their staining in the normal animal. Recovery was quickest in the structures which were still slightly stained 30 min after poisoning. The progression of the AChE staining in these structures from the perikaryon to the nerve fibers was not easily observed. In some normally lesser stained structures, the recovery of AChE staining seemed to occur later and the progression of the staining from the neuronal perikarya to the corresponding nerve fibers was detectable on sections from rats killed early and at short time intervals. This was particularly visible in the following structures: the ventro-lateral nucleus of the thalamus in plane I; all the AChE containing structures, except for the nucleus interpeduncularis in plane II; and the reticular formation in planes III and IV.

In some normally lightly stained structures, the recovery of the AChE staining seemed to appear even later. Such is the case for the cerebral cortex and for the hippocampus.

Determination of ChE Activity

The mean values of the ChE activity measured in normal animals showed that the striatum contains about four times more ChE than the cerebellum and the neocortex and twice as much as the medulla, the mesencephalon and the entorhinal cortex.

The measures carried out after poisoning revealed for every CNS area a large individual variability in the degree of early inhibition and in the recovery of enzymatic activity in the following days. In spite of this variability, variance analysis showed that, in general, the level of inhibition and the time course of recovery of ChE vary from one structure to another. Figure 9 shows that soman poisoning produced a rapid enzymatic inhibition, the level of which is different in the six areas. About 90 min after poisoning, the mean inhibition as compared to normal rats was 77% in the striatum, 64% in the mesencephalon, 60% in the

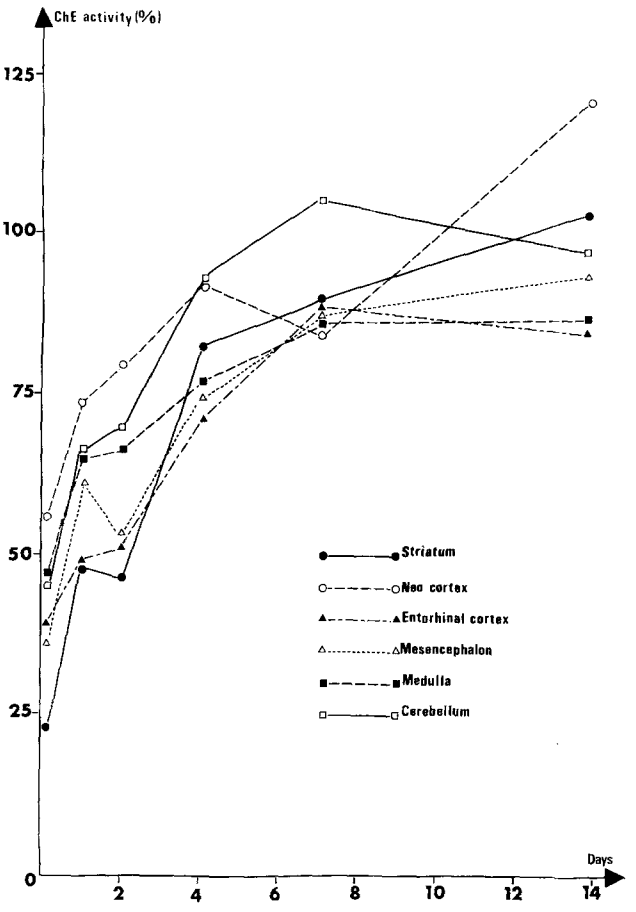


Fig. 9. The time course of activity of the ChE (in percent of the mean enzymatic activity of the controls) in six regions of the rat CNS following s.c. soman poisoning with 100 µg/kg. According to the large individual variations of the measures the standard deviations (SD) of the mean are not indicated

entorhinal cortex, 55% in the medulla and the cerebellum, and 44% in the neocortex. The enzymatic activity increased considerably after 24 h in every structure except for the entorhinal cortex. The enzymatic increase was fast in all structures until the 4th day, then it became slower. In the striatum, the most inhibited region, three quarters of the lost enzymatic activity were recovered after 4 days, whereas only about 50% of the ChE activity reappeared in the same time in the medulla, the mesencephalon, and the entorhinal cortex, which were not so severely inhibited. The ChE level in the examined structures seemed still reduced even 8 days after poisoning, though there was no significant difference with the normal mean values.

We searched for a relation between the severity of symptoms and the initial enzyme inhibition. In 12 rats killed 24 or 48 h after poisoning, moderate or severe symptoms were generally associated with a strong enzyme inhibition in most of the studied areas. Two rats which had not exhibited detectable symptoms manifested a weaker inhibition of ChE. The mean level

of enzyme inhibition was, however, not higher in rats with severe symptoms than in rats with moderate ones.

Discussion

The experimental intoxication of rats with a single s.c. injection of about one LD_{50} of soman induced, in most of the surviving animals, neuronal lesions reproducing the picture of experimental anoxic-ischemic encephalopathy of the rat (Brown and Brierley 1968), comparable to the hypoxic damage of human CNS. Our experiments revealed that this encephalopathy was not observed in every rat given the same dose of soman. Its presence was not directly related to the amount of injected toxic material. However, neuronal lesions occurred in animals surviving for more than 6 h and having exhibited severe and prolonged respiratory distress and iterative and persistent convulsions. It is well known that each of these syndromes may cause in man as in animals (Meldrum and Brierley 1973) similar damage to the CNS. Therefore, the encephalopathy observed in rat after soman poisoning probably results at least in part from an hypoxic state of the CNS. It must be stressed that recent experimental studies (Söderfelt et al. 1981) tend to show that the early electron-microscopic neuronal alterations due to status epilepticus would be different from those of hypoxia. An ultrastructural study of early neuronal damage following OP poisoning in rat is in progress in our laboratory. It will perhaps help to clear up this problem.

Our histochemical observations in the normal rat are consistent with those of Palkovits and Jakobowitz (1974) and of Jakobowitz and Palkovits (1974). They demonstrated that the structures known as cholinergic, i.e., the anterior horns and the intermediolateral nuclei of the spinal cord and the motor nuclei of cranial nerves, as well as the structures considered as cholinergic in the "ascending cholinergic reticular system" of Shute and Lewis (1967) and the "cholinergic limbic system" of Lewis and Shute (1967) were not affected by neuronal lesions. On the contrary, several structures designated as cholinoceptive by these authors, i.e., the cerebral cortex, the hippocampus, the medial and lateral geniculate bodies, the thalamus, the amygdaloid nucleus, were often severely affected. There were also sometimes a few lesions in the striatum, which seems to be both cholinergic and cholinoceptive. Therefore, the supposed excessive release of ACh produced by the inhibition of AChE could participate in the process of degeneration of cholinoceptive neurons.

The results of the measures of ChE activity are mostly in accordance with the histochemical observations: rapid inhibition of the AChE by soman in all selected samples, reappearance of the AChE activity as early as 24 h after poisoning, followed by fast increase of the AChE level, especially in the structures the richest in the enzyme (e.g., the striatum), incomplete recovery of the normal AChE activity at day 8. Jovic (1974) previously mentioned that the normal level of the brain ChE was not reached 21 days after a severe soman poisoning of the rat. The relative correlation of the level of ChE inhibition with the severity of the symptoms is not very conclusive owing to the small number of animals.

In rats chronically exposed to soman, the delayed occurrence of the acute symptomatology at the 4th injection may be explained by the summation of several successive incomplete AChE inhibitions, which may lead to an enzyme decrease sufficient to trigger the cholinergic crisis.

The encephalopathy observed in rats experimentally poisoned with soman may be expected, in case of survival, to produce long-lasting behavioral and electroencephalographic changes, depending on the localization and the spread of neuronal destruction. Such lesions could account for some long-term effects on the human CNS following acute OP poisoning, when mechanical ventilation allows the patient to survive. The somatic basis of the neuropsychologic and electroencephalographic alterations mentioned in some human cases of chronic OP intoxication occurring without severe cholinergic crisis ought to be further experimented with electrophysiology, neurobehavioral tests, and biochemistry.

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Received February 2, 1983/Accepted May 2, 1983