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ACCUMULATION OF ACETYLCHOLINE IN BRAIN AND BLOOD OF ANIMALS POISONED WITH CHOLINESTERASE INHIBITORS

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The extreme toxicity of a group of phosphorus compounds, of which tetraethylpyrophosphate (TEPP) and di*iso*propylfluorophosphonate (DFP) are examples, is attributed to their powerful inhibition of cholinesterase. Substances of this type cause generalized cholinergic stimulation, convulsions, and neuromuscular paralysis, effects which are thought to be due to accumulation of acetylcholine at certain nerve endings throughout the body. The object of the experiments reported here was to discover whether abnormal amounts of acetylcholine were present in animals poisoned with such compounds.

METHODS

Acetylcholine was determined by means of the heart of *Venus mercenaria,* isolated according to the procedure of Tower and McEachern (1948). The isolated heart was mounted in an organ bath containing 10 ml. of Venus saline of the following composition : 30 g. NaCl, 5.1 g. $MgSO_4.7H_2O$, 0.9 g. KCl, 0.14 g. $NaH_2PO_4.H_2O$, 0.95 g. $CaCl_2$, 0.25 g. glucose, and 0.0001 g. phenol red per litre, together with sufficient 0.1 M-Na_2HPO_4 to adjust the *p*H to 7.2. The solution was stirred and oxygenated by a slow stream of air bubbles.

The spontaneous contractions of the isolated Venus ventricle were recorded with a 10-inch counterweighted gimbal lever, at tenfold magnification with a load of 0.6 g., on smoked paper moving at the rate of 0.12 mm. per sec.

Doses of standard acetylcholine bromide solution and of the solution to be assayed were added to the bath alternately at four-minute intervals, allowed to act for 70 sec., and then removed by washing out the organ bath with three changes of fresh saline. Responses were obtained for two dosage levels of both standard and unknown, twice repeated, according to a conventional two-dose assay plan, described by Bliss and Marks (1939). All timing and washing operations were performed automatically by an electric cycle timer.

The depressions of the amplitude of the recorded heartbeat were measured and expressed as a percentage of the amplitude of the normal beat (taken as a mean of the amplitude of the beat before the dose and after recovery from it). The percentage

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responses were transformed to probits, and the calculation of the concentration ratio between standard and unknown completed by the method of Bliss and Marks (1939).

In preliminary experiments rat brain was ground with sand, with addition of 0.3 mg. TEPP in 0.1 ml. propylene glycol, 1.5 ml. 0.33 N-hydrochloric acid, and 1.5 ml. Venus saline. The homogenate was transferred to a centrifuge tube, allowed to stand for 30 minutes or one hour, centrifuged, and the supernatant solution decanted and neutralized for assay. The tissue residue was mixed with additional quantities of TEPP, acid, and Venus saline as before, and allowed to stand for a second extraction period. The second supernatant solution was also neutralized and assayed. In practice it was found that at least three such extractions were necessary : thus in one experiment two extractions of 1 hr. each gave $0.74 + 0.34 = 1.08$ μg. acetylcholine per g. brain ; in a second experiment three extractions of 30 min. each gave 1.08 (first two extractions) $+ 0.16 = 1.24$ μg.

When the tube containing the tissue homogenate was shaken continuously, a considerable speeding up of the extraction resulted. The results of such an experiment, comparing opposite halves of the same rat brain, extracted with and without shaking, were as follows : Amounts of acetylcholine in μg. per g. brain obtained in four extractions of 30 min. each : (i) with shaking : $2.56 + 0.84 + 0.50 + 0.14 = 4.04$; (ii) without shaking : $1.49 + 0.99 + 0.96 + 0.47 = 3.91$; a fifth extraction gave amounts too small for accurate measurement.

When the homogenate was placed in a boiling water bath for five minutes, for the first extraction only, and then shaken continuously for 30-minute periods as before, the extraction was slightly faster. It was decided to include this step in the procedure in order to prevent any enzymic accumulation or destruction of acetylcholine in the extracts. Three extractions with continuous shaking were considered to be enough, because a fourth would contain less than 10 per cent of the acetylcholine already extracted, an increment which approximates the limits of accuracy of the biossay for acetylcholine.

The procedure finally adopted for making brain extracts was as follows : the rat was killed (by the particular method selected), and the whole brain dissected out, including the cerebellum and a variable proportion of the medulla oblongata. The tissue was blotted free of blood, weighed, and transferred to a mortar with 0.3 mg. TEPP in 0.1 ml. propylene glycol, 1.5 ml. 0.33 N-hydrochloric acid, 1.5 ml. Venus saline, and a sufficient quantity of washed sand. The brain was rapidly and thoroughly ground to a paste. The homogenate was transferred to a centrifuge tube with the addition of three 0.5 ml. portions of Venus saline. The tube was immersed in a boiling water bath for five minutes, with occasional shaking. The tube was cooled, stoppered, and shaken by machine, in a longitudinal direction for thirty minutes. The tube was centrifuged, and the supernatant fluid decanted ; 1.5 ml. 0.33 N-hydrochloric acid and 1.5 ml. Venus saline were added to the tissue residue, and shaking repeated for a second 30-minute period. After centrifugation, the supernatant was removed, and the tissue residue was extracted for a third time. The combined extract was brought to *p*H 6.8 by cautious addition of 0.33 N-sodium hydroxide solution in the presence of a drop of 0.1 per cent phenol red. The extract was slightly reacidified with one drop of 0.33 N-hydrochloric acid, and stored in the frozen state. For assay, the solution was thawed out and diluted to 25 ml. with Venus saline.

In the first experiment, rats were killed in groups of four, one by each of the four methods : (1) asphyxia in nitrogen gas containing 5 per cent carbon dioxide ; (2) intraperitoneal injection of 1 mg. strychnine sulphate per kg. ; (3) chloroform inhalation ; (4) intraperitoneal injection of 2 mg. TEPP per kg. The order of killing was systematically rotated according to a Latin square design in order to avoid any bias due to the order of killing.

Brains were extracted and assayed on the day on which the rats were killed, a single Venus mercenaria heart preparation being used. The whole procedure was repeated on four different days, thus completing a 4 × 4 Latin square.

In the second experiment the same methods of killing and preparation of brain extracts were used, but the extracts were stored until all the rats had been killed ; assays were then done on groups of four extracts, the groups taken vertically through the Latin square, so that four extracts assayed together represented the four methods of killing but taken on successive groups of rats.

In the third experiment control rats were killed by asphyxia, as before. The three other rats in each group were killed by intraperitoneal injection of 8.0 mg. DFP per kg. ; 4 mg. Paraoxone (diethyl *p*-nitrophenyl phosphate) per kg. ; or 3 mg. eserine sulphate per kg. The Latin square design was completed in the same way as the second experiment, and the extracts were stored an average of six days. Eight extra animals were included to provide additional data.

Results

Comparison of the effect of TEPP poisoning on the acetylcholine content of rat brain, with those of asphyxia, chloroform anaesthesia, and strychnine poisoning

The object of this experiment was to find whether TEPP poisoning caused an increase in the acetylcholine content of rat brain, as might be expected on theoretical grounds. To do this necessitated a comparison of brains from TEPP poisoned animals and from " normal " control animals. The nature of the " normal " control group posed a problem, because physiological factors are known to affect the acetylcholine content of the brain (Richter and Crossland, 1949). Certain physiological disturbances associated with TEPP poisoning could alter the acetylcholine content of the brain, without the change being associated directly with the specific anticholinesterase action of TEPP. For example, convulsions occur during TEPP poisoning, and Elliott, Swank, and Henderson (1950) have shown that convulsants decrease the acetylcholine content of the brain. Furthermore, anoxia, which invariably accompanies severe TEPP poisoning, is also known to lower the acetylcholine content (Welsh, 1943). It was decided to use two groups of controls, one group killed by a lethal dose of the convulsant strychnine, the other asphyxiated in an atmosphere of nitrogen containing 5 per cent of carbon dioxide ; these methods of killing would seem to approximate to the conditions to be expected during TEPP poisoning, apart from the specific anticholinesterase effect. However, in order not to load the experiment too heavily in favour of the expected result, a third group of rats were killed with chloroform. Anaesthesia increases the acetylcholine content of brain (Elliott, Swank, and Henderson, 1950), and consequently it was thought that, if the acetylcholine content of the brains of TEPP poisoned rats was greater than that of anaesthetized animals, the increase could be regarded as outside " normal " limits, and attributable to the specific cholinesterase inhibiting action of TEPP.

The results of these two experiments are shown in Table I. Within each assay, acetylcholine was present in greater quantity in the brain extract from the TEPP poisoned animal than from any of the controls. The differences between the means for different treatments, as tested by Fisher's " t " test applied to paired data, are all statistically significant, except that between asphyxia and strychnine.

The assays of extracts in the second experiment show considerably lower values compared to the first. The difference is attributed to destruction of acetylcholine during storage in the second experiment; the mean storage period was approximately ten days.

TABLE I

ACETYLCHOLINE FROM BRAINS OF RATS, KILLED BY ASPHYXIA, STRYCHNINE, CHLOROFORM, AND TEPP

	μg. Acetylcholine per g. brain			
	Asphyxia	Strychnine	Chloroform	TEPP
First experiment				
Assay No. 1	3.2	2.3	2.2	4.4
2	3.3	2.2	3.3	4.1
3	6.6	6.8	6.9	10.7
4	2.8	3.1	2.3	4.6
	Means 3.97	3.60	3.67	5.98
Second experiment				
Assay No. 1	1.2	2.4	3.5	3.4
2	1.1	1.0	1.7	2.3
3	1.5	1.8	2.5	2.5
4	1.9	1.1	1.6	2.7
	Means 1.42	1.57	2.32	2.72
Grand	Means 2.69	2.58	3.00	4.35

Analysis of variance applied to the data revealed a highly significant difference between assays done with different Venus hearts. This phenomenon, which was also encountered by Welsh (1943), is difficult to understand, although it does not affect the main conclusion of the experiment. Several possible causes may be postulated, all invoking differential responses by different Venus hearts to some substance or substances in brain extracts other than freely dissolved acetylcholine.

The results of the third experiment are shown in Table II. In this experiment differences between the effects of the different cholinesterase inhibitors are relatively unimportant, because they may merely reflect differences in the arbitrary doses used. The important point is their common property of increasing the acetylcholine content of the brain above the physiological range. The differences between the results from the asphyxiated controls and each of the three groups killed with cholinesterase inhibitors were statistically significant.

Appearance of free acetylcholine in blood of animals poisoned with cholinesterase inhibitors

The previous experiments show that when rats are killed with cholinesterase inhibitors, more acetylcholine can bé extracted from their brains than when they are killed by asphyxia or anaesthesia. It seems likely that, when the cholinesterase is

TABLE II

ACETYLCHOLINE FROM BRAINS OF RATS, KILLED BY ASPHYXIA, DFP, PARAOXONE, AND ESERINE

	μg. Acetylcholine per g. brain			
	Asphyxia	DFP	Paraoxone	Eserine
Assay No. 1	1.7	3.7	3.7	3.4
2	2.1	2.3	2.2	1.4
3	0.5	2.3	4.4	2.5
4	1.8	4.5	1.6	1.6
5	2.8	9.6	—	7.3
6	1.7	3.0	7.3	5.0
	Means 1.77	4.23	3.84	3.53

inhibited, acetylcholine accumulates at synapses throughout the brain, causing widespread stimulation of neurones, followed by paralysis. It should be pointed out, however, that the acetylcholine content of these extracts does not necessarily represent free acetylcholine in the brains from which the extracts were made. The acetylcholine in normal brain is probably almost entirely " bound " to brain cells in some form ready to be released upon the arrival of nerve impulses. The cholinesterase content of brain is so great that free acetylcholine must exist only very briefly and in small quantities.

If free acetylcholine does indeed accumulate in the brain and other tissues during anticholinesterase poisoning, it should appear in the blood. The following experiments demonstrate that this occurs.

Rats were given lethal doses (4 mg. per kg. intraperitoneally) of paraoxone. When signs of severe intoxication appeared, but before respiration failed, the rats were decapitated, and blood was collected. The blood was mixed with 50 units of heparin and 0.05 mg. paraoxone, to prevent clotting and loss of acetylcholine. Control rats were lightly anaesthetized with ether, decapitated, and blood collected in the same way.

The blood samples were immediately tested for the presence of acetylcholine by adding 1.0 ml. quantities of the whole blood to a 10 ml. bath containing an isolated *Venus mercenaria* heart, which had been sensitized by previous treatment with DFP.

Five pairs of rats were used. In every pair blood from the control rats either contained no detectable acetylcholine, or a very small amount, always less than 0.005 μg. per ml. blood. The blood samples from rats poisoned with paraoxone all contained definite amounts of acetylcholine, varying from 0.008 to 0.03 μg. per ml. blood. The kymograph tracing from a typical experiment is shown in Fig. 1.

In a single experiment, a rhesus monkey was anaesthetized with sodium pentobarbital. Venous blood samples taken during anaesthesia contained no detectable acetylcholine. DFP was given intraperitoneally in divided doses to a total of 4 mg. per kg. Acetylcholine was detected in a final blood sample taken just as respiration ceased, but in none of the previous samples. The kymograph record is shown in Fig. 2.

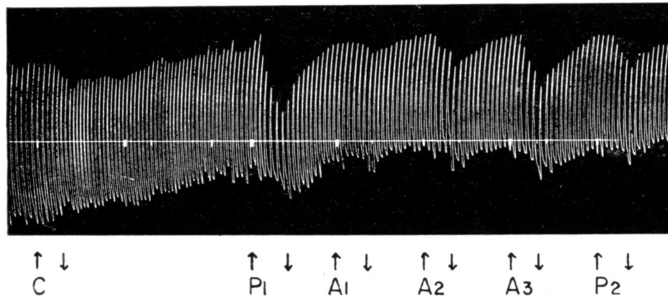

FIG. 1.—Isolated *Venus mercenaria* heart in 10 ml. bath, sensitized by previous addition of DFP ↑ indicates additions; ↓ indicates washes. At C, 1.0 ml. of whole heparinized blood (containing added paraoxone) from a rat killed by light ether anaesthesia and decapitation. The slight diminution in amplitude is accompanied by acceleration of the beat. At P_1, 1.0 ml. of whole heparinized blood (containing added paraoxone) from a rat killed by paraoxone and decapitation. The marked diminution in amplitude is accompanied by slowing of the beat. At A_1, A_2, and A_3, standard acetylcholine bromide in amounts of 0.005, 0.010, and 0.020 μg. respectively. At P_2, 0.5 ml. of the same blood sample used at P_1.

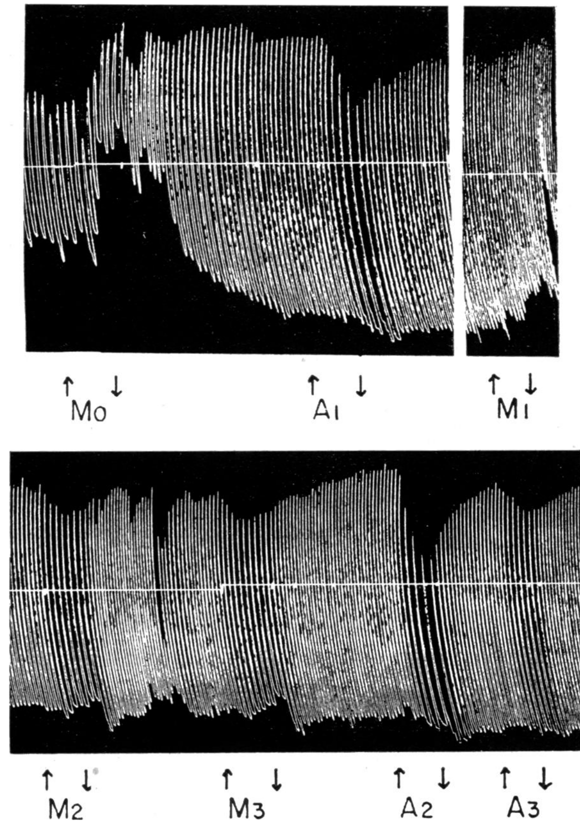

FIG. 2.—Isolated *Venus mercenaria* heart in 10 ml. bath, sensitized by previous addition of DFP. ↑ indicates additions; ↓ indicates washes. At M_0, 1.0 ml. of whole heparinized blood (containing added DFP) from a rhesus monkey anaesthetized with sodium pentobarbital. The diminution of amplitude is apparently due to failure to relax between beats, and is accompanied by acceleration of the beat. At A_1, 0.010 μg. acetylcholine bromide. At M_1, 1.0 ml. of blood from the same monkey, 30 min. after intraperitoneal injection of 1.0 mg. DFP per kg. No parasympathomimetic effect is visible. At M_2, 1.0 ml. of blood taken after a total dose of 4.0 mg. DFP per kg., and at the time of respiratory paralysis. The diminution of amplitude is accompanied by slowing, an effect typical of acetylcholine. At M_3, the same as M_2. At A_2 and A_3, 0.010 and 0.005 μg. respectively of acetylcholine bromide.

Summary

1. Lethal doses of TEPP increased the total acetylcholine content of brains of rats over that of rats killed by asphyxia, chloroform, or strychnine.

2. DFP, paraoxone, and eserine also caused an increase in total acetylcholine content of rat brain.

3. During severe anticholinesterase poisoning, free acetylcholine appeared in the blood.

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