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ACETYLCHOLINE PRODUCTION IN ANIMALS POISONED BY DIETHYL-*p*-NITROPHENYL PHOSPHATE (PARAOXON)

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During a study of the toxic action of diethyl-*p*-nitrophenyl phosphate (paraoxon, E600), which is a powerful inhibitor of cholinesterase (Aldridge, 1950), it was found that anaesthetized fully atropinized rabbits could be revived from an otherwise lethal dose by artificial respiration, and that they then became insensitive to further injections of the same dose of this inhibitor (Barnes, 1953). It was noted that the animals given paraoxon remained very sensitive to the effects of ACh and that this sensitivity increased temporarily after each dose of inhibitor. If death from failure of respiration after the first dose of the inhibitor could be attributed to the action of excessive amounts of ACh in the brain or at other important sites, the insensitivity of the anaesthetized rabbit to subsequent doses might result from a reduced ACh production in the poisoned animal.

Douglas and Paton (1951), using an inhibitor with very similar properties (TEPP, tetra-ethyl-pyrophosphate), found that levels of 40 mμg. ACh/ml. were reached in the blood of the cat after a large single dose. They stated that much of this ACh came from the bowel. Stewart (1952), using the isolated *Venus mercenaria* heart, assayed the blood of rats dying from paraoxon poisoning and found it to contain 8–30 mμg. ACh/ml. ACh was "just detectable" in the blood of monkeys at the time respiration failed after poisoning with di*iso*-propyl phosphofluoridate (DFP, dyflos). More recently, Perry (1953) has amplified the work of Brown and Feldberg (1936), who found that ACh production from the perfused eserinized cervical sympathetic ganglion of the cat fell rapidly during periods of continued stimulation. Perry found that this fall in output did not occur in the absence of eserine. This suggested that a prolonged inhibition of cholinesterase might be associated with a diminished production of ACh.

The experiments to be described were designed to test these hypotheses.

METHODS

Male rabbits (1.5–3 kg.) were anaesthetized with urethane (1 g./kg.) given intravenously as a 25% solution. Male and female dogs (9.5–16 kg.) and cats (2.5–3.5 kg.) were anaesthetized with pentobarbitone sodium (30 mg./kg., i.v.).

In all animals a tracheal cannula was inserted and the carotid blood pressure was recorded from a Hg manometer. A record of the respiratory movements was obtained by a thread tied to the lower part of the chest. To obtain blood for ACh assay, a polythene cannula was introduced into the superior (rabbits) or inferior (dogs and cats) vena cava from a peripheral vein.

Blood samples (7–10 ml.) were withdrawn into 0.2 ml. of 1% heparin, and eserine (10^{-5} M) was added immediately after. The assays were carried out within a few minutes.

All drugs were given intravenously. Atropine was given freely except in experiments where the dose was deliberately kept as low as possible. It was given before the first injection of paraoxon and with each dose; it was also given between doses if the condition of the animal suggested that it might be beneficial. The total dose administered ranged from 2–7 mg./kg. Paraoxon was prepared as a solution in alcohol and injected as a 1:10 emulsion made by diluting this in atropine solution immediately before injection.

The isolated rat diaphragm preparation was essentially that described by Bülbring (1946), with the small bath (7 ml.) suggested by Burgen, Dickens and Zatman (1949). The diaphragm was stimulated by supramaximal rectangular impulses of 0.2 msec. duration at a frequency of 50/sec. for 20 min., and the contents of the bath then immediately removed. When the assays were not done at once, this fluid was acidified with HCl to *p*H 4, frozen at $-15°$ C., and kept so until just before assay. Eserine (5×10^{-5} M) or paraoxon (4×10^{-6} M) was added to the bath at least 5 min. before stimulation was started.

ACh in the blood was assayed on the leech dorsal muscle. Normal blood had little or no effect on this preparation. At least 7 ml. blood was required for each assay; this limited the number of samples that

could be taken from a single animal. The ACh levels were expressed as millimicrograms (mμg.) base per ml. blood. Fluid from the diaphragm bath was assayed on the blood pressure of the chloralosed cat. The techniques and precautions described by MacIntosh and Perry (1950) were followed:

RESULTS

Rabbits.—Rabbits were given successive doses of 0.5 mg./kg. paraoxon at intervals of 30 min. and their response was similar to that already described (Barnes, 1953); the animals died with progressive respiratory and cardiac failure after three or four doses. The ACh level in the blood was determined 10–15 min. after each dose of paraoxon; the levels (mμg./ml. whole blood) in five animals are given in Table I.

TABLE I

BLOOD LEVELS OF ACh (MμG. BASE/ML. WHOLE BLOOD) IN ATROPINIZED RABBITS 15 MIN. AFTER SUCCESSIVE DOSES OF PARAOXON 0·5 MG./KG.

Interval between doses 40–45 min.

Dose Sequence	Rabbit No.				
	1	2	3	4	5
1st dose	4·6	26	12	9	16
2nd „	18·6	35	—	16	—
3rd „	28·0	44	16	30	26
4th „	28·0	—	34	43	—

There was always a progressive rise in the blood ACh. In one rabbit the level was followed for 90 min. before giving a further dose of paraoxon; in this period it fell to about 50% of the level attained just after the inhibitor had been given.

Dogs.—The experiments were continued on dogs because they could provide a greater number of blood samples for assay. In four animals the initial dose of paraoxon was 3 mg./kg. and this caused temporary respiratory failure which was relieved by a short period (2–3 min.) of artificial respiration. One dog was given a larger initial dose (10 mg./kg.), but it died in spite of artificial respiration. The animals that survived the initial dose of paraoxon were quite unaffected by subsequent doses of up to 20 mg./kg., and no dog died of the effects of paraoxon alone, though some were observed for as long as 7 hr., and received total doses of 40–70 mg./kg. They remained sensitive to ACh, and an intravenous injection of 500 μg. would arrest respiration and kill them.

The blood levels in four dogs 30 min. after receiving successive doses of paraoxon are given in Table II. The blood ACh levels rose after the first and second doses of paraoxon, but thereafter there was little further change. In one animal (Dog 3, Table II) the blood level was followed for 4 hr. after the second dose of paraoxon; it fell from 20 mμg./ml. to 15 mμg./ml. at 3 hr., and to 10 mμg./ml. at 4 hr. A third dose of paraoxon then raised it promptly to 20 mμg./ml. The rate of fall of the blood ACh is so slow that slight variation in the interval between doses makes little difference to the result. The blood levels reached did not depend upon the amount of inhibitor given.

In two animals serial determinations of the blood cholinesterase were carried out by a standard manometric method. The total blood cholinesterase, with ACh as substrate, was reduced to less than 5% of normal and did not vary significantly throughout the 5-hr. periods of the experiments.

In another dog the blood ACh was raised to 30 mμg./ml. by giving 5 successive doses of paraoxon. Measured quantities of ACh were then injected intravenously and the blood levels determined immediately, and again 2 hr. later. The blood level after injection corresponded closely to that which would have been expected had the same quantity been added to a volume of blood equivalent to 6% of the body weight. The rate of dis-

TABLE II

BLOOD LEVELS OF ACh (MμG. BASE/ML. WHOLE BLOOD) IN ATROPINIZED DOGS 30 MIN. AFTER SUCCESSIVE DOSES OF PARAOXON

Interval between doses was 45–50 min., except in dog 3, where it was 4 hr. between the 2nd and 3rd doses

Dog No.		Dose Sequence						
		1st	2nd	3rd	4th	5th	6th	Total
1	Paraoxon mg./kg.	3	5	5	10	20	—	43
	ACh mμg./ml.	15	25	20	20	15	—	
2	Paraoxon mg./kg.	3	5	5	5	10	10	38
	ACh mμg./ml.	25	30	30	30	25	20	
3	Paraoxon mg./kg.	3	5	5	10	—	—	23
	ACh mμg./ml.	10	20	20	25	—	—	
4	Paraoxon mg./kg.	3	5	10	10	20	20	68
	ACh mμg./ml.	10	15	20	25	20	20	

appearance from the blood remained slow even when the level had become high (120 mμg./ml.). These findings suggest that the persistent low level of ACh in the blood of dogs poisoned by paraoxon is due to a slower production of ACh rather than to an increased rate of excretion or destruction.

Cats.—Since Douglas and Paton (1951), and Perry (1953), used cats for their observations on ACh output, further experiments were done on these animals. The cats' response to an initial dose of paraoxon (3 mg./kg.) was a failure of respiration similar to that seen in dogs and rabbits ; but a more prolonged period of artificial respiration was usually necessary before natural breathing returned—an average of 17 min., as compared with 2–3 min. in dogs. The response of cats to a second dose of paraoxon was uncertain. Some, like dogs, were unaffected ; others showed further failure of respiration needing prolonged artificial respiration before recovery. Most of the cats died from the effects of the paraoxon, developing circulatory failure in addition to respiratory failure. However, they survived for periods of up to 5½ hr., and observations on the blood ACh levels were made. Cats responded like rabbits (Table III) and showed a progressive rise in the blood ACh. The figures were usually obtained about 30 min. after the paraoxon had been given. On two occasions, when samples were taken 5 min. after the paraoxon, the level was a little higher (by about 5 mμg./ml.) than it was 25 min. later.

The progressive rise of blood ACh only took place when successive doses of paraoxon were given. If these were discontinued the blood level fell slowly, decreasing by about 50% in 2 hr. There is no direct relationship between the total amount of inhibitor given and the final blood level of ACh.

Douglas and Paton (1951) stated that much of the blood ACh in cats poisoned with TEPP came from the intestine. When our experiments were repeated on eviscerated cats a progressive rise of blood ACh took place, but at a reduced rate (Table IV). Two of these cats received smaller initial doses of paraoxon, but otherwise the experiments were comparable to those on normal cats.

In the hope that it might eventually prove possible to analyse blood from individual organs or regions of the body, an attempt was made to assay small samples of blood by the chloralosed cat method. It was found that the doses of atropine normally given to the poisoned cat interfered with the assay.

TABLE III

BLOOD LEVELS OF ACh (MμG. BASE/ML. WHOLE BLOOD) IN ATROPINIZED CATS 30 MIN. AFTER SUCCESSIVE DOSES OF PARAOXON

Interval between doses was 40–45 min., except in cat 3, where it was 2 hr. between 2nd and 3rd doses

Cat No.		Dose Sequence						
		1st	2nd	3rd	4th	5th	6th	Total
1	Paraoxon mg./kg.	3	5	10	13	—	—	31
	ACh mμg./ml.	15	25	45	75	—	—	
2	Paraoxon mg./kg.	3	5	5	5	5	—	23
	ACh mμg./ml.	15	30	35	45	60	—	
3	Paraoxon mg./kg.	3	5	10	10	20	20	68
	ACh mμg./ml.	0	10	20	30	35	45	
4	Paraoxon mg./kg.	3	5	10	10	10	—	38
	ACh mμg./ml.	20	30	40	60	70	—	

TABLE IV

BLOOD LEVELS OF ACh (MμG. BASE/ML. WHOLE BLOOD) IN EVISCERATED ATROPINIZED CATS AFTER SUCCESSIVE DOSES OF PARAOXON

Interval between doses 40–45 min.

Cat No.		Dose Sequence								
		1st	2nd	3rd	4th	5th	6th	7th	8th	Total
1	Paraoxon mg./kg.	1	2	3	5	5	5	5	5	31
	ACh mμg./ml.	—	10	13	20	25	30	45	56	
2	Paraoxon mg./kg.	1	3	5	10	10	10	—	—	39
	ACh mμg./ml.	—	10	15	20	20	40	—	—	
3	Paraoxon mg./kg.	3	5	10	10	10	10	17	—	65
	ACh mμg./ml.	5	7·5	7·5	15	20	25	35	—	

An attempt was made to reduce the amount of atropine to the minimum compatible with survival. The prophylactic atropine was omitted, but a dose of 0.5 mg. was given after the first dose of inhibitor (1 mg./kg.). Further doses of atropine were given as successive doses of paraoxon were injected. To three cats, which received 0.5–1.0 mg./kg. of atropine, a total of 11–16 mg./kg. paraoxon was given.

All the cats died of paraoxon poisoning with blood levels of 20–35 mμg./ml. ACh as determined on the leech. But these levels were in the range at which it was just possible to use the chloralosed cat method of assay; and, despite the smaller doses of atropine that had been given, enough was still present to invalidate the assay.

Rat Diaphragm.—Perry (1953) found that it was the inhibition of the cholinesterase in the perfused eserinized cat sympathetic ganglion that led to the profound fall in ACh output in response to continued stimulation. The fall did not occur after stimulation in the absence of eserine. The observations on the cat and rabbit poisoned with paraoxon suggested that ACh production might continue after, and be "stimulated" by, repeated doses of the inhibitor. Since it proved impossible to assay the blood from individual organs of the poisoned cat, ACh production in the isolated rat diaphragm was examined. The anaesthetized rat responds like the rabbit or dog to repeated doses of paraoxon, and Stewart (1952) has shown that ACh is present in its blood before death from paraoxon poisoning. Burgen, Dickens and Zatman (1949) showed that the stimulated isolated rat diaphragm will produce ACh in measurable quantities.

When the diaphragm was stimulated at 50/sec. for 20 min. the fluid in the bath contained no ACh. If eserine (5×10^{-5} M) was added, in order to inhibit all the cholinesterase in the preparation, and the diaphragm was stimulated for a period of 20 min., then from 40–100 mμg. of ACh were produced. When four successive periods of stimulation were applied to single preparations there was no consistent or significant decrease in the amount of ACh produced. It made no difference whether the interval between periods of stimulation was 5 min. or 40 min. Some of the figures obtained in these experiments are given in Table V; it will be seen that eserine and paraoxon gave similar results.

The production of ACh seemed to be steady throughout the period of stimulation, and the amount produced was proportional to the total number of stimuli applied—whether these were in increasing or decreasing order of frequency.

TABLE V

ACh BASE (MμG.) PRODUCED BY A HALF DIAPHRAGM AFTER SUCCESSIVE 20 MIN. PERIODS OF TETANUS AT 50/SEC.

Rest intervals between tetanus periods are either 5 min. or 40 min. Vol. of sample from diaphragm bath = 5 to 7 ml. Max. range of each ACh value = ±25%.

Period of Tetanus	Eserine 5×10^{-5}M						Paraoxon 4×10^{-6}M					
	5′ rest interval			40′ rest interval			5′ rest interval			40′ rest interval		
1st ..	55	50	41	55	54	40	60	30	90	54	52	90
2nd ..	46	55	55	50	50	45	50	32	60	30	62	60
3rd ..	40	57	27	30	50	50	50	0*	50	59	59	43
4th ..	46	46	50	50		50	60	32	50	60	57	65

* Values of 0 were very occasionally recorded with paraoxon, but never with eserine.

Thus the diaphragm produced 22, 35, and 50 mμg. ACh when the sequence of stimulation was 10, 20, and 50 per sec. respectively; and it produced 65, 32 and 22 mμg. ACh when the sequence was 50, 20, and 10 stimuli per sec.

DISCUSSION

In the anaesthetized rabbit, dog, and cat the failure of a second dose of paraoxon to inhibit respiration and kill the animal is not associated with a lower level of ACh in the blood. In the cat and the rabbit successive doses of paraoxon produce a progressive rise in the blood ACh; but if no further doses are given the blood level slowly falls, and is reduced by 50% after 2–3 hr. In the dog the ACh level does not continue to rise after the third or succeeding doses of inhibitor; if no more inhibitor is given the level falls slowly, as in the cat. The dog is much less sensitive to successive doses of inhibitor than is either the cat or the rabbit, though it remains sensitive to injected ACh—which will induce a prolonged and fatal arrest of respiration.

The tolerance of the cat to the circulating ACh is probably related to the amount of atropine administered, which also appears to circulate for some time. The cats given smaller doses of atropine died earlier than those given larger quantities. Protection by atropine is limited, however, and cannot be indefinitely increased by larger doses.

Could the insensitivity of the anaesthetized animal to succeeding doses of paraoxon be due to a falling off in the production of ACh, such as Perry (1953) had shown to take place in the perfused and eserinized sympathetic ganglion of the cat? In our experiments the level of ACh in the circulation only was measured. This blood ACh is almost certainly an overflow ACh from the tissues, and is the balance between the amount entering the blood stream, and the sum of that

destroyed by any remaining cholinesterase, plus any unchanged ACh which leaves the blood. In two experiments the cholinesterase activity of dogs' blood was consistently less than 5% of normal. The methods of estimation were not accurate enough to determine whether the activity of the cholinesterase of the cat was lower than that of the rabbit. The blood level of ACh does not rise so high in the dog as it does in the rabbit or cat; the dog is also much less sensitive to repeated doses of the inhibitor.

In the absence of further doses of inhibitor the rate of disappearance of ACh from the blood was similar in the three species. Judged by the blood levels, ACh production is most rapid within the first few minutes of giving the inhibitor.

The differences between the cat and rabbit on the one hand, and the dog on the other, could most easily be explained by assuming that the production of ACh is less in the dog; but other explanations are possible.

Death from paraoxon poisoning may be due to excess ACh at certain vital centres whose activities are suppressed or distorted by it. Such an excess may result from a high local production, or from the carriage to such sites of ACh produced elsewhere. Although Douglas and Paton (1951) found the bowel to be an important site for ACh production our observations on eviscerated cats indicate that other tissues produce at least as much as does the gut.

From the work of Perry (1953) it seemed likely that ACh production might fall off very much after prolonged inhibition of cholinesterase such as would follow the large doses of paraoxon given in these experiments. However, in the stimulated isolated diaphragm, ACh production does not fall off, after inhibition of cholinesterase, as it does in sympathetic ganglia. On the hypothesis put forward by Perry to explain his observations one would assume that at sites such as the myoneural junction there was plenty of raw material available for the continuous production of ACh.

On the assumption that death does take place as a result of excess ACh at certain points, an explanation of the findings recorded above, and of those of Perry (1953), can be suggested. At or near certain vital points which are sensitive to ACh, there is an excessive ACh production in response to an initial "lethal" dose of a cholinesterase inhibitor such as paraoxon. If, however, the circulation is maintained by artificial respiration this excess is carried away by the blood, and the animal recovers. But at such sites ACh production falls off rapidly in the presence of continuously inhibited cholinesterase, just as it does in the perfused sympathetic ganglion. Meanwhile ACh production continues at other sites, such as the myoneural junction, and is apparently "stimulated" by each further addition of inhibitor. Production at such sites goes on more rapidly in the rabbit and cat than in the dog, so that in the first two species the blood levels continue to rise until the vital centres are again overwhelmed—this time by ACh brought by the blood. This does not happen in the dog, because its blood ACh never rises as high as that of the cat or rabbit. That the dog is not insensitive to ACh is shown by its response to large doses injected into the blood.

It is difficult to understand the rise of ACh in the blood of cats and rabbits in terms of a true stimulation of production. Nothing is yet known about any such stimulus. It seems probable that a temporary more complete inhibition of cholinesterase takes place. It is not, however, technically possible to measure the small differences in cholinesterase activity that may be important in these conditions.

ACh will protect cholinesterase from inhibition by this type of inhibitor, but to demonstrate this phenomenon it has always been necessary to use concentrations of ACh of an order several times greater than those of the inhibitor (Aldridge, 1950; Augustinsson and Nachmansohn, 1949). This makes it seem unlikely that the protection of any residue of inhibited cholinesterase by ACh is responsible for the insensitivity of the animals to the second and subsequent doses of inhibitor. Were such a mechanism of protection important it would be the cat rather than the dog that would display the greater degree of insensitivity to repeated doses of paraoxon.

Summary

1. The blood level of acetylcholine has been followed in anaesthetized rabbits, dogs, and cats poisoned by the anticholinesterase diethyl-*p*-nitrophenyl phosphate (paraoxon) and kept alive by atropine and short periods of artificial respiration.

2. In the rabbit and cat there is a progressive rise in the blood level of ACh if successive doses of inhibitor are given. If no more inhibitor is given the level falls slowly, to about one half in two hours. Cats and rabbits may die because of respiratory and circulatory failure despite atropine and artificial respiration.

3. In the dog the blood level rises, but not so far as in the cat or rabbit, and the rise is not progressive after repeated doses of inhibitor. The

dog seems to become immune to paraoxon though it remains sensitive to injected ACh. The rate of fall in the absence of further inhibitor is even slower than in the cat or rabbit.

4. The blood ACh in the poisoned eviscerated cat rises progressively though more slowly than in the normal cat.

5. The ACh produced by the stimulated isolated rat diaphragm, in the presence of eserine or paraoxon, remains steady over a number of successive periods of stimulation.

6. These findings are discussed, and an explanation offered for them.

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