Effects of Two Chlorinated Phenols on the Spontaneous Impulse Activity of the Abdominal Tonic Motor System in the Crayfish (*Astacus Fluviatilis* L.)

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Chlorinated and many other substituted phenols are widely used as nonspecific pesticides (BEVENUE and BECKMAN 1967, MELNIKOV 1971), but little information is available on their effects on physiological functions in animals, and invertebrates in particular. Chlorophenols, especially pentachlorophenol, have been shown to act as uncouplers of oxidative phosphorylation in mitochondria (WEINBACH 1956a, 1956b, WEINBACH and GARBUS 1965, PARKER 1958). This mechanism readily explains the universal toxicity of these compounds, but there is not enough information to show whether it is the ultimate cause of mortality in animals due to chlorophenol intoxication.

One aim of the present study was to find out some clues on the basic modes of action of pentachlorophenol (PCP) and 2,3,6-trichlorophenol (TCP) by observing their effects on the abdominal tonic motor system of the crayfish. Another purpose was to test the usefulness of this preparation for further neurotoxicological work. The physiological phenomenon used as the indicator in these experiments was the spontaneous impulse activity of the tonic system (KENNEDY et al. 1966) recorded from the roots of an isolated ganglion.

Experimental results achieved *in vitro* are often difficult to evaluate in a practical context, so a parallel study has been made of the acute toxicity of PCP and 2,3,6-TCP to the crayfish (KAILA and SAARIKOSKI 1976).

MATERIALS AND METHODS

The experimental animals

The crayfish (*Astacus fluviatilis* L.) were caught in an unpolluted lake in Southern Finland in autumn, and kept in a large steel container with constantly flowing dechlorinated tap water at a temperature of

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4 ± 1°C. The weight of the crayfish ranged from 20 to 30 g. Liver and raw carrot were used for feeding. The crayfish were adapted to the experimental temperature, 13°C, for two to three weeks. The experiments were performed in January and February.

The preparation

The abdominal tonic motor system of the crayfish controls all the postural and slow movements of the abdomen (KENNEDY and TAKEDA 1965a, 1965b, KENNEDY et al. 1966, KOVAC 1974). Six flexor and six extensor neurons located in each of the six abdominal ganglia send their axons through a ventral (posterior) superficial branch of the third root and through a dorsal superficial branch of the ganglionic second root, respectively, to the tonic flexor and extensor muscles (KENNEDY and TAKEDA 1965a, KENNEDY et al. 1966). The amplitudes of the extracellularly recorded unit action potentials differ according to the axon diameters.

Arrangement of the experiments

The abdomen of the crayfish was removed and placed in ice-cold saline for dissection. The abdominal cord was isolated with a lenght of the appropriate roots intact, and one of the first five abdominal ganglia was transferred to a 30 x 10 x 10 mm polyethylene recording chamber. The others, used later, were kept in cold (4°C) aerated saline, where they remained in good condition for at least 24 hours. A series of experiments on one cord took 3 - 5 hours.

The recording chamber was located in a thermostated water bath and received a constant flow through a polyethylene tube connected via a three-way tap to two bottles containing the control and experimental solutions. A few loops of this tube were placed in the water bath to adjust the temperature of its contents. The bottles were chilled and continuously aerated to ensure air saturation.

The temperature was adjusted to 13°C by aid of a thermocouple in the recording chamber, connected to a potentiometric pen recorder. The maximum fluctuation of temperature was less than ±0.05°C during any single experiment. No temperature changes were observed when changing from control to experimental solution or vice versa.

Action potentials of one superficial third (flexor) root and one ganglionic second (extensor) root were recorded simultaneously with glass suction electrodes

(e.g. FLOREY and KRIEBEL 1966) connected to a Tektronix 5103N/D13 storage oscilloscope and to two loudspeakers via Tektronix 122 preamplifiers, and stored on separate tracks of an AM tape recorder. The activity of the preparation was allowed to settle to a steady level at the beginning of each experiment. This took 15 - 30 minutes, after which the test solution was conducted to the recording chamber.

Quantification of spontaneous activity

The devices used for measuring the impulse frequencies of single axons consisted of monostable multivibrators, whose output pulses (1 ms) were fed into separate RC-tachometer circuits with time constants of 6 s. The triggering levels of the multivibrators were adjusted between the spike amplitudes of different axons. The output voltages of the tachometers were serially subtracted, so that the final output voltages were linearly proportional to the impulse frequencies of axons with different spike amplitudes. In some cases the spikes of two units differed so little in amplitude that their summed frequencies were measured. The circuits were constructed from LM 3900 quad operational amplifiers (see the application manuals of National Semiconductors).

The recordings from both roots were simultaneously analysed with two analysers in order to correlate the frequency changes in the spontaneously active axons. As both the compounds under study affected the amplitude of the action potentials, the amplification of the input signals from the tape recorder had to be adjusted from time to time to ensure that the spikes exceeded the initial triggering levels.

Chemicals

The crayfish saline used was van Harreveld's (1936) solution, with 8 mM glucose and a 10 mM Tris buffer added. Its pH was adjusted with HCl to 7,8 at the experimental temperature. This pH corresponds to that of Astacus hemolymph (DITTMER 1961). The pentachlorophenol (purissimum grade, Fluka AG) and 2,3,6-trichlorophenol (purum grade, Fluka AG) were further purified by recrystallizing them three times from a mixture of ethanol and water, and dissolved in an equivalent amount of 1 N NaOH.

RESULTS

Effects on impulse frequencies

PCP affected the impulse frequencies of the tonic motor axons in concentrations above 1 ppm, and TCP in concentrations above 10 ppm. In both roots, the frequency changes usually consisted of an inhibition of the smaller, higher-frequency units, with a concomitant excitation of the bigger units. In many cases, antagonistic and synergistic relationships between the activity of different units were clearly noticeable (see Figs 1, 2 and 3). No differences were found between the sensitivities of the second root and third root units.

Judging from the relative spike amplitudes, neither compound excited the fast extensor axons located in the second ganglionic root.

The frequency changes were phasic, i.e. the maximum response was usually attained soon after the application of the test solution. A clear transient of this kind lasted 2 - 5 minutes on the average. At the lowest effective concentrations (PCP: 1 - 2 ppm; TCP: 10 - 20 ppm) the response amplitude was smaller, and its phasic nature was less pronounced (e.g. Fig. 3).

The latency of the response was remarkably short, only 10 - 15 s, in the highest concentrations tested (PCP: 10 ppm; TCP: 100 ppm). Owing to the phasicity of the frequency changes, the effect of rinsing with control saline was sometimes difficult to evaluate, but in many cases rinsing clearly reversed the changes (e.g. Figs 2 and 3).

To compare quantitatively the potencies of the two compounds, an EC50 assay was made, on a quantal basis. The occurrence of a frequency change in at least one of the units of a given preparation served as the criterion. The results are shown in Table 1. Estimates of the EC50 values and the potency ratio were obtained by the method of LICHTFIELD and WILCOXON (1949): The EC50 estimate was 1.5 ppm (95 % confidence limits: 1.0 - 2.2) for PCP and 14 ppm (8.5 - 23) for TCP, corresponding to 5.6×10^{-6} M and 7.1×10^{-5} M. The estimate of the potency ratio was 9.3 (5.0 - 17) on a weight basis, indicating the stronger activity of PCP.

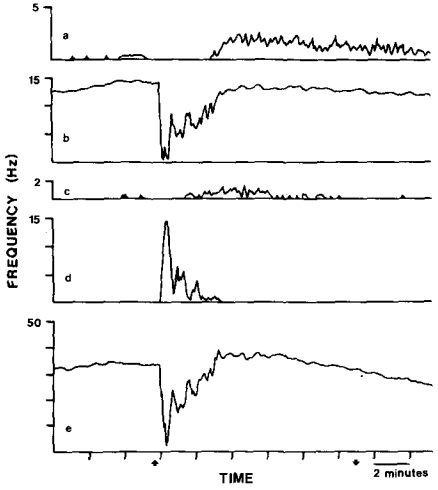

Fig. 1. Effect of 50 ppm TCP on the tonic impulse activity of a third abdominal ganglion. a: big flexor unit; b: small flexor unit; c: big extensor unit; d: big extensor unit (smaller than c); e: summed activity of two small extensor units. Arrow upwards: start of application; arrow downwards: start of rinsing.

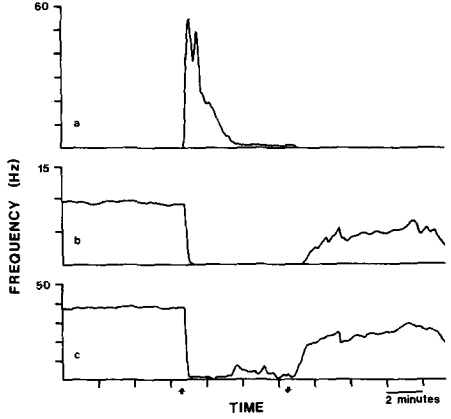

Fig. 2. Effect of 100 ppm TCP on the tonic impulse activity of a first abdominal ganglion. a: big flexor unit; b: small flexor unit; c: summed activity of two small extensor units.

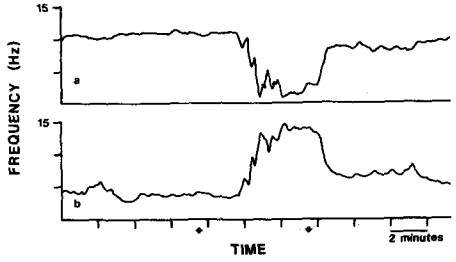

Fig. 3. Effect of 2 ppm PCP on the tonic impulse activity of a fourth abdominal ganglion. a: small flexor unit; b: small extensor unit.

TABLE 1

Results of the EC50 assays

Concentration (ppm)	No. of preparations affected / No. of preparations tested	
	PCP	TCP
1	1/6	-
2	3/4	-
5	6/6	-
10	5/5	1/4
20	-	3/4
50	-	5/5
100	-	5/5

Effects on action potentials

Both the compounds diminished the amplitudes and increased the durations of the extracellularly recorded action potentials, sometimes leading to conduction block in the higher concentrations. (Inactivation of this kind was not regarded as a frequency change in the sense implied in the previous section.)

In this case, too, PCP was found to be more effective than TCP. The action potentials in the third superficial root were much more sensitive to the action of the two compounds than those in the second ganglionic root, which is understandable because the former is very thin (50 - 80 µm). The latencies and sensitivities of the action potential deformations were similar to those of the frequency changes.

In most cases the effects were completely reversible. The effect of rinsing began after a latency of 15 s or more.

DISCUSSION

The preparation used showed several advantages in this kind of toxicological work. Its activity remains stable for many hours and is rather easy to quantify. Owing to the complexity of the preparation its activity can be expected to be sensitive to various biologically active compounds, as was shown to be the case with chlorophenols.

An explanation for the prolonged duration and suppressed amplitude of the action potential after PCP and TCP application is that the compounds may interfere with the energy metabolism of the nerve cells, thereby affecting the resting membrane potential and, consequently, the excitation cycle. This explanation is supported by the fact that chlorophenols act as uncouplers of oxidative phosphorylation in mitochondria (WEINBACH 1956a, 1956b, WEINBACH and GARBUS 1965, PARKER 1958). However, the rapid onset of the changes, as well as their rapid reversal, observed in our experiments, do not fit this hypothesis well. An alternative hypothesis is that the compounds have a direct effect on the conductance changes related to the action potential, as do some other phenolic compounds with well established uncoupling properties (COOKE et al. 1968, BARKER and LEVITAN 1971). - The question will be examined in a future study by means of intracellular recordings.

The similarities in the latencies and in the effective concentrations of the chlorophenols affecting the action potential and impulse frequencies suggest that the mechanism underlying both phenomena is basically the same, and the frequency changes are thus due to a chlorophenol-induced depolarization of some excitable cell membranes belonging to the tonic motor system. A direct effect on the chemical aspects of synaptic transmission cannot, however, be excluded (cf. KUBA 1969, on phenol). Because the frequency changes in different axons were clearly coupled, the effect must involve neurons on a higher level than the spontaneously active motoneurons, since the latter do not show strong synaptic interactions (TATTON and SOKOLOVE 1975).

Since chlorophenols are weak acids, the potency ratio estimate obtained is valid only at the pH of 7.8 used in the present experiments (BLACKMAN et al. 1955,

SIMON and BEEVERS 1952). As this is the pH of Astacus blood, PCP could be expected to be more toxic than PCP to crayfish tissues in vivo. This agrees with the results of an LD50 assay demonstrating the greater toxicity of PCP (KAILA and SAARIKOSKI 1976).

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