Bioalkylation of Nucleic Acids in Mice by Insecticides*
I. Alkylation of Liver RNA and DNA by Chlorpyrifos

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Z. Naturforsch. **38 c**, 461 – 464 (1983); received September 22, 1982

DNA, RNA, Alkylation, Insecticides, Chlorpyrifos

Male mice were i.p. injected with two doses (5 & 15 mg/kg body weight) of [1-^{14}C]ethyl-labelled chlorpyrifos. The radioactivity in liver, fat, kidney and urine was measured 6, 24, 48, and 192 h after treatment. Liver RNA and DNA were isolated and hydrolyzed and the bases separated by ion-exchange chromatography. Radioactivity resulting from incorporation and alkylation of RNA and DNA was measured. Labelled 7-ethylguanine was found in RNA hydrolysate which amounted 5.5×10^{-3} calculated as fraction of the applied dose. In DNA hydrolysate [^{14}C]7-ethylguanine was missing and the major radioactivity was found in two unknown peaks, one of which corresponds to 3×10^{-2} of the applied dose. The results indicate that the extent of alkylation obtained with clorpyrifos is high as compared with other organophosphates with methyl esters.

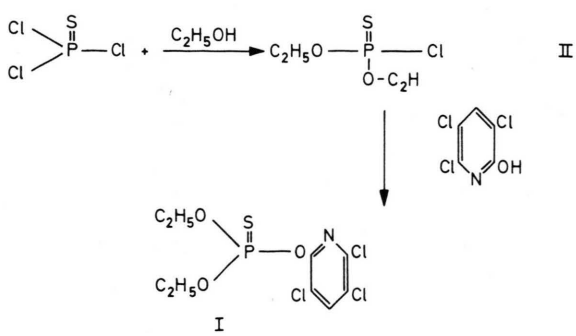
In Egypt, a variety of pesticides are extensively used to increase the yield of economic crops. Chlorpyrifos (dursban; O,O-diethyl-O-3,5,6-trichloro-2-pyridyl phosphorothioate) is one of the most commonly used insecticides in Egypt for the control of cotton leaf worm [1, 2]. Recently, it has been demonstrated that chlorpyrifos induces a high percentage of polychromatic erythrocytes with micronuclei [3] and chromosome breakage [4] in mouse-bone marrow following intraperitoneal and oral administration. It also induces a significant percentage of cells with chromosome aberrations in root-mitosis of *Vicia faba* plants [5]. This prompted us to conduct a series of studies on the chemistry and toxicology of this important insecticide. A number of organophosphorus insecticides were classified as alkylating agents among which some were found to be weak carcinogens [6].

The present investigation deals with the *in vivo* alkylating properties of chlorpyrifos towards N-7 of guanine in nucleic acids in mice.

Materials and Methods

Synthesis of ^{14}C-labelled chlorpyrifos

[1-^{14}C]ethanol (6 mmol, 1 mCi) was allowed to react with thiophosphoryl chloride (3 mmol) in presence of pyridine to give O,O-diethyl thiophosphorochloridate (**II**). After removal of the separated pyridine hydrochloride, the reaction mixture was taken in benzene and extracted with 1 N HCl to remove the rest of pyridine. After removal of the solvent, the residue was allowed to react with the sodium salt of 3,5,6-trichloropyridinate in acetone to give [1-^{14}C]ethyl-labelled chlorpyrifos (**I**). The crude product was purified by chromatography on silica gel column using benzene for elution. The



Synthesis of Chlorpyrofos

* This work was supported in part by U.S. Environmental Protection Agency, Environmental Toxicology Division, Toxic Effects Branch. Triangle Park. N.C. 27711 under Research Agreement No. 3-545-6.

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0341-0382/83/0500-0461 $ 01.30/0

radiopurity was determined by TLC radiotracer technique and by inverse isotope dilution; yield of pure chlorpyrifos 670 mg of sp. act. 0.83 mCi/g; $R_f = 0.8$ in benzene : methanol : acetic (39 : 14 : 7) and 0.73 in hexane : benzene (9 : 1).

Animals

Male mice weighing about 30 g and 15 weeks of age were given standardized food pellets. A solution of the labelled insecticide in 0.1 ml dimethyl-sulphoxide was injected intraperitoneally. The doses/animal used during this study were 150 and 450 µg (corresponding to 5 and 15 mg/kg body weight) respectively. At different time intervals the animals were sacrificed by chloroform and organs were immediately removed and stored at − 20 °C till analysis. These included liver, fat, kidney as well as urine.

Analysis:

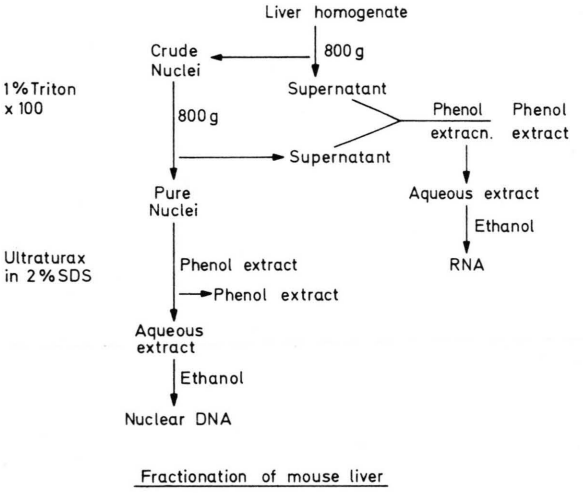
a) Isolation of Nucleic acids

1. Extraction of total cell RNA

The total cell RNA was obtained by phenol extraction using a modified method which is based on the techniques described earlier [7−9]. The livers were homogenized in 0.03 M phosphate buffer (pH 6.8) containing 0.14 M NaCl. The homogenate was centrifuged at $800 \times g$ for 10 min. An equal volume of water saturated with phenol was then added to the supernatant, stirred for one hour after which a 0.5% sodium dodecylsulphate (SDS) was added and the mixture was allowed to stand in a water bath at 65 °C for 15 min. After standing overnight, the mixture was centrifuged at $250 \times g$ for one hour for better separation of the two phases. The aqueous layer was then subjected to centrifugation at $1100 \times g$ to get rid of any traces of protein for 20 min. It was then extracted with ether and RNA was separated by the addition of two volumes of ethanol and allowed to stand over night at − 20 °C.

2. Extraction of nuclear DNA

The crude nuclear pellet was homogenized in 10 ml 1% Triton X-100. After centrifugation at $800 \times g$ for 10 min, the pellet was resuspended in 10 ml Tris-sucrose buffer (0.05 M Tris HCl, pH 7.6, 0.025 M KCl, 0.005 M $MgCl_2$ and 0.25 M sucrose) and recentrifuged. The pellet was finally suspended in 5 ml 2% SDS-solution, homogenized (ultraturax) and incubated for 1 h at 40 °C. Ten ml phosphate buffer (0.03 M, pH 6.8, containing 0.14 M NaCl) and 15 ml of a mixture of phenol, chloroform and isoamyl-alcohol (50 : 50 : 1, v/v) were added and the mixture was stirred for 30 min and left over night at room temperature. After centrifugation, the upper (aqueous) layer was extracted three times with an equal volume of ether. DNA was then precipitated from aqueous layer by the addition of 2 volumes of ethanol as in RNA (Scheme).



Fractionation of mouse liver

b) Base analysis

Each nucleic acid was dissolved in 6 ml phosphate buffer (pH 6.8) and the solution was fortified with guanine, 7-ethyl-guanine and adenine (0.2 mg each). Hydrolysis was performed by heating with 1N HCl for 1 h at 100 °C. The hydrolsate was fractionated on anion exchange column of Dowex 50 W X−12 (H^+) (100−200 mesh), equilibrated with 1 N HCl by gradient elution from 1−4 N HCl (200 ml each, 40 ml/h). The fractions (5 ml each) were measured using SP 6−400 UV-spectrophotometer PYE-UNICAM at 260 µm. The samples were evaporated under reduced pressure and the residues taken up in 0.2 ml distilled water and 2 ml methanol. The latter was added to avoid acid quenching.

c) Distribution studies

For the determination of ^{14}C-activity in different organs, one gram tissue (liver, fat or kidney) as well

as 1 ml urine were oxidized in a Packard Sample Oxidizer (306). The samples were then counted using a Nuclear Chicago Liquid Scintillation Spectrometer Model 194. The scintillation cocktail used consisted of permablend (5.5 g) and naphthalene (120 g) in one liter dioxane.

Results and Discussion

The distribution of ^{14}C-activity among liver, fat, kidney and urine at different times following intraperitoneal injection of the ^{14}C-labelled chlorpyrifos is shown in Table I. The amount of radioactivity excreted in urine shows a rapid rate of elimination of the insecticide and/or its metabolites. The ^{14}C-activity in liver and fat reached its maximum value after 6 h following application and decreased thereafter. This may indicate that chlorpyrifos does not tend to accumulate in these tissues.

The amount of ^{14}C-activity found in the liver and fat decreased with time, while that in the kidney showed a constant increase of chlorpyrifos and/or its metabolites. This is in agreement with the findings on rats fed with [^{36}Cl]Chlorpyrifos [10]. Whereas the acute toxicity of organophosphates is mainly due to their phosphorylation properties, *i.e.*, ability to inhibit cholinesterase enzymes, the late-time effects or the chronic toxicity might be caused by alkylation of certain bases in the nucleic acids. The alkylation properties of organophosphorus insecticides on nucleic acids were recognized in the last few years [11], and the chemical and biochemical significances of alkylation reactions were summarized [12–14]. We now have found that the ^{14}C-incorporation in nucleic acids, prepared from mouse liver, was greater in RNA than in DNA; and with the 15 mg dose than with 5 mg/kg body weight (Fig. 1).

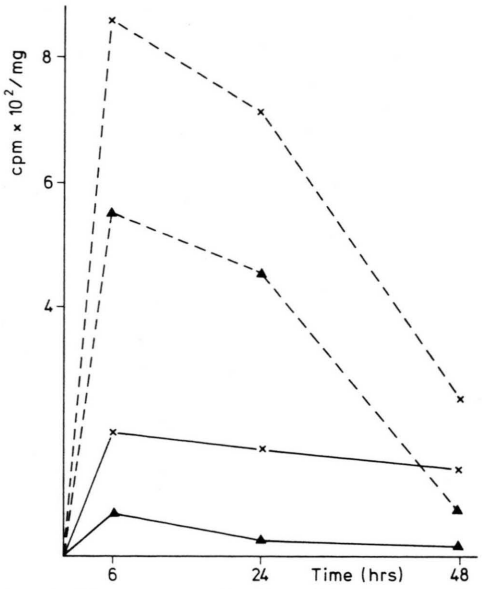


Fig. 1. Time courses of radioactivity in mouse liver nucleic acids after intraperitoneal administration of [^{14}C]chlorpyrifos (5 mg and 15 mg/kg body weight).
RNA ×---× DNA ▲---▲ 15 mg/kg
RNA ×—× DNA ▲—▲ 5 mg/kg

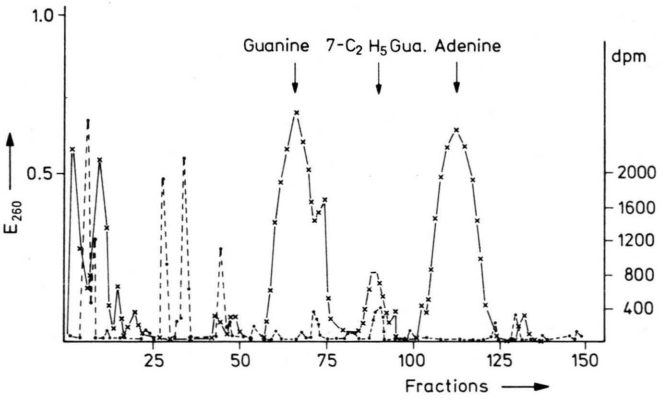


Fig. 2. Chromatographic and radioactive profile of acid hydrolysis of mouse liver RNA via ion exchange chromatography on Dowex 50 (H) eluted with 1–4 N HCl. Guanine, 7-ethyl-guanine and adenine were added (0.2 mg each) as tracers before hydrolysis. ×—× = E_{260}; ○---○ = ^{14}C-activity. Data mean of duplicate experiments.

Table I. Amount of ^{14}C (μg/g tissue) in liver, fat, kidney and urine (μg/ml) following i.p. administration of ^{14}C-labelled chlorpyrifos to male mice.

Time [h]	Tissue							
	Liver		Fat		Kidney		Urine	
	D_1	D_2	D_1	D_2	D_1	D_2	D_1	D_2
6	12.2	20.8	22.3	29.4	2.9	5.1	–	–
24	8.7	16.2	8.9	22.8	3.2	7.1	46.5	91.7
48	7.8	15.0	5.0	22.8	5.7	19.4	50.9	120.7
192	5.6	9.7	4.1	14.7	12.9	30.6	20.2	31.7

D_1 = initial dose 150 μg/mouse.
D_2 = initial dose 450 μg/mouse.

It can be shown that the amount of radioactivity in both acids decreases rapidly with time. A similar trend has been observed with other organophosphorus insecticides, such as trichlorphon, where a

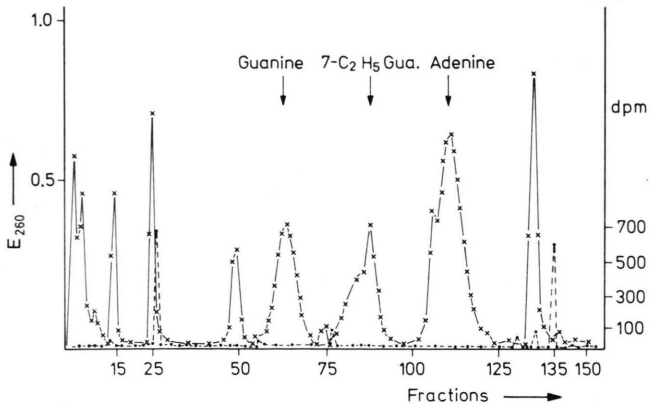


Fig. 3. Ion exchange chromatography and radioactive profile of mouse liver DNA-hydrolysate after treatment with chlorpyrifos. Chromatography and elution were performed as in RNA. x—x = E_{260}; ○---○ = ^{14}C-activity. Data mean of duplicate experiments.

half-life of 2 h was calculated for the removal of [7-^{14}C]methyl guanine from mouse liver DNA and RNA following i.p. administration [15]. By base hydrolysis and choice of specific targets, *e.g.*, 7-alkylated guanine, it would be possible to discriminate between incorporation via C-1 pool and alkylation. Analysis of labelled RNA-hydrolysate by chromatography on Dowex 50 revealed the presence of a radioactive peak coinciding with the UV-peak of 7-ethyl guanine (Fig. 2), and amounting to 4% of total RNA-radioactivity. This peak corresponded to 5.5×10^{-3} of the applied dose. These results clearly indicate the presence of 7-ethylguanine, since the N-7 of guanine was found to be the preferable site of alkylation in nucleic acids [15]. The corresponding peak, however, could not be detected in case of labelled DNA (Fig. 3). The major part of radioactivity in the latter (64%) was obtained in two peaks: the first at fractions 25–27 (36% and corresponds to 3×10^{-2} % of the applied dose) and the second at fractions 139–141 (28%). The identification of these peaks is still under further investigation. The extent of alkylation which could be achieved with chlorpyrifos was fairly high if compared with trichlorphone and other organophosphates with methyl esters [15]. The latter compounds, however, are known to possess higher alkylating properties than esters with long-chained alkyl groups.

[1] A. A. Kmel, M. Mitri, and H. Samina, J. Econ. Entomol. **63,** 609 (1970).
[2] A. S. M. Marie, A. S. A. Saad, and G. Tantawy, J. Agric. Sci **82;** 391 (1974).
[3] S. M. Amer, O. R. Farah, and M. A. Fahmy, Europ. J. Cell Biol. **22,** 571 (1980).
[4] S. M. Amer and M. A. Fahmy (personal communication).
[5] S. M. Amer and O. R. Farah, Cytologia (Jaman), (1981) Under Publi.
[6] R. D. Kimbrough and T. B. Gaines, Arch. Environ. Health, **16,** 805 (1968).
[7] M. Eto and H. Ohkawa, Biochemical Toxicology of Insecticides, (R. D. O'Brien, ed.), p. 93 Academic Press, New York 1970.
[8] H. S. Rosenkranz and S. Rosenkranz, Experientia **28,** 386 (1972).
[9] S. Rosenkranz, Cancer Res. **33,** 458 (1973).
[10] G. N. Smith, B. S. Watson, and F. S. Fischer, J. Agric. Food Chem. **15,** 132 (1967).
[11] R. Preussmann, Food Cosmet. Toxicol. **6,** 576 (1968).
[12] K. Scherrer and J. E. Darnell, Biochem. Biophys. Res. Comm. **7,** 486 (1962).
[13] G. B. Georgiev and V. L. Mantieva, Biochem. Biophys. Acta. **61,** 153 (1962).
[14] K. S. Kirby, Biochem. J. **64,** 405 (1956).
[15] W. Dedek, Kh. Lohs, G. W. Fischer, and R. Schmidt, Pesticide Biochem. Physiol. **6,** 101 (1976).