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A COMPARATIVE STUDY OF PORPHYRIN ACCUMULATION IN TISSUE CULTURES OF CHICKEN EMBRYO HEPATOCYTES TREATED WITH ORGANOPHOSPHOROUS PESTICIDES

ALAN W. NICHOL and LYNDALL A. ANGEL
School of Applied Science, Riverina College of Advanced Education, P.O. Box 588, Wagga Wagga, N.S.W. 2650, Australia

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Abstract—Thirty-two organophosphorous pesticides have been examined for their ability to cause porphyrin accumulation in cultures of chicken embryo hepatocytes. Greatest porphyrin accumulation is associated with compounds which have an aromatic leaving group and are phosphate or thionophosphate ethyl esters. Compounds of this type cause accumulation of uroporphyrin in the medium. Paroxon and diazoxon cause inhibition of uroporphyrinogen decarboxylase in the cells. The ability to cause accumulation of uroporphyrin in the medium does not correlate with the alkylating properties of the compound as measured by its rate of alkylation of 4-(*p*-nitrobenzyl)-pyridine.

The association of some organochlorine compounds with porphyria cutanea tarda is well known. In recent years special interest has been shown in the chlorodioxins [1], and the polychlorinated biphenyls [2]. Porphyria cutanea tarda is relatively common in rural areas of Australia. Some cases of the disease appear to be associated with exposure to the organophosphorous insecticide, diazinon [3, 4]. Recently, a case of the disease was seen where an association could be demonstrated with a close structural analogue of diazinon, pyrimiphos-ethyl [5]. It was therefore of interest to examine a range of similar compounds for their ability to interfere with porphyrin biosynthesis. Although its relevance to effects on whole animals may need to be treated with caution, the chicken embryo hepatocyte system of Granick [6] was used as a convenient screening method.

MATERIALS AND METHODS

Most organophosphorous compounds were gifts from the manufacturer [7]. Diazoxon was prepared as follows. 2-Isopropyl-6-methylpyrimid-4-one (2 g) was dissolved in dry methanol (20 ml) containing sodium methoxide (0.8 g). After evaporation and drying, the sodium salt of the pyrimidone was suspended in acetonitrile (20 ml). Diethyl chlorophosphate (1.68 g) was added and the mixture heated at 75° for 2 hr. After addition of dichloroethane (20 ml), the mixture was washed with water (4 ×), dried over K_2CO_3 and evaporated to dryness. The remaining colourless oil (2.2 g) showed n.m.r., mass spectra and u.v. spectra consistent with the structure of diazoxon. Paraxon was prepared using the same molar quantities and conditions from 4-nitrophenol and diethyl chlorophosphate. Isodiazinon was prepared by the method of Nichol *et al.* [4] and isoparathion by an analogous method from 4-nitrothiophenol. Cells for tissue culture were derived from chicken embryos eighteen days after fertilisation and were cultured in Williams medium E using a modified method of Sassa and Kappas [8]. Embryos were perfused through the heart with calcium and magnesium free Hank's solution and were disaggregated by incubation with collagenase (Sigma Type IV, 5 mg/ml) and hyaluronidase (Sigma IVS, 4 mg/ml) in Williams medium E. The cells were grown either in 24-well Linbro boxes (for screening) or on 3.5 cm Petri dishes (for enzyme assays) on a layer of rat tail collagen. The collagen was prepared by the method of Wood and Keech [9]. The layers were prepared by addition of 0.02 ml of a 1 mg/ml solution of collagen in isotonic saline to a 3.5 cm dish and 0.01 ml of solution per well in a Linbro box. After air drying at 20° the dishes were sterilised under u.v. light and were used within 2 hr. Cells were cultured for the first 24 hr in medium containing 5% foetal calf serum. After this period the medium was replaced with Williams medium E. About 1 mg of the organophosphorous compound was dissolved in 0.1 ml of ethanol which was diluted with sterile water immediately prior to addition to the cultures. 0.01 ml of this emulsion was added to each culture to give a final concentration of 65 μM. The cultures were incubated with the organophosphorous compounds for 24 hr, diluted with an equal volume of 0.5 M perchloric acid in methanol and allowed to stand for 15 min. Porphyrin concentrations were determined fluorimetrically using coproporphyrin as standard. Each compound was tested in at least ten wells or dishes. Analyses did not vary by more than 10% between wells. Protein determinations were carried out by the Lowry method on the cell monolayer using two separate wells per dish. Cells were

Abbreviation: ALA, δ-aminolaevulinic acid.

Table 1. Porphyrin accumulation in culture and alkylation potential of organophosphorous compounds

Organophosphorous compound	Porphyrin accumulation (pmoles hr/mg protein)		Rate of alkylation of 4-(*p*-nitrobenzyl) pyridine (ΔA_{554} min^{-1}) × 10^3 at 80°	Porphyrin type in absence of ALA (number of carboxyl groups) (trace amounts in parentheses)
	Medium only	Medium plus 500 μM ALA		
AROMATIC LEAVING GROUP				
Paroxon diethyl 4-nitrophenyl phosphate	3,411	2,459	0.6	8, 7
Diazoxon diethyl 6-methyl-2-(1-methylethyl)-4-pyrimidinyl phosphate	2,675	2,490	0.1	8, 7
Zinophos O,O-diethyl O-pyrazinyl phosphorothioate	1,646	2,498	9.3	8, 7
Bromophos-ethyl O-(4-bromo-2,5-dichlorophenyl) O,O-diethylphosphorothioate	1,397	1,929	4.6	8, 7
Isodiazinon O,O-diethyl S-[6-methyl-2-(1-methylethyl)-4-pyrimidinyl phosphorothioate	1,098	1,965	15.2	(8), (7), 4
Pyrimiphos ethyl O-[2-(diethylamino)-6-methyl-4-pyrimidinyl] O,O-diethyl phosphorothioate	871	1,765	3.3	8, 7
**Azinphos methyl* O,O-dimethyl S-[(4 oxo-1,2,3-benzotriazin-3(4H)-yl)-methyl]phosphorodithioate	537	1,218	6.3	(8), (7), 4
**Azinphos ethyl* O,O-diethyl S-[4 oxo-1,2,3-benzotriazin-3(4H)-yl)-methyl] phosphorodithioate	520	1,330	10.0	8, 7
Dowco 214 O,O-dimethyl O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate	447	1,416	3.0	4
Parathion O,O-diethyl O-(4-nitrophenyl) phosphorothioate	426	1,301	8.0	4
Fenthion O,O-dimethyl O-[-3-methyl-4-(methylthio) phenyl] phosphorothioate	391	1,463	13.6	8, 7, 6, 4
Chlorphyrifos O,O-diethyl O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate	351	1,966	11.3	8, 7
Cyanophos O-(4-cyanophenyl) O,O-dimethyl phosphorothioate	341	1,476	16.6	4
Carbophenothion S-[[(4-chlorophenyl)thio]methyl] O,O-diethyl phosphorodithioate	339	1,207	5.3	4

Fenchlorphos O,O-dimethyl O-(2,4,5-trichlorophenyl) phosphorothioate	325	1,366	24.6	4, 2
Isoparathion O,O-diethyl S-(4-nitrophenyl) phosphorothioate	305	1,475	21.2	4
Fenitrothion O,O-dimethyl O-(3-methyl-4-nitrophenyl) phosphorothioate	294	1,285	16.0	8, 7
Idofenphos O-(2,5-dichloro-4-iodophenyl) O,O-dimethyl phosphorothioate	274	1,465	42.6	8, 7
Diazinon O,O-diethyl O-[6-methyl-2-(1-methylethyl)-4 pyrimidinyl] phosphorothioate	250	1,671	15.2	(8), (7), 4
Dicapthon O-(2-chloro-4-nitrophenyl) O,O-dimethyl phosphorothioate	248	1,133	40.6	4, 2
Pirimiphos O-[2-(diethylamino)-6-methyl-4-pyrimidinyl] O,O-dimethyl phosphorothioate	146	1,292	62.6	(8), (7), 4
Fospirate dimethyl 3,5,6-trichloro-2-pyridinyl phosphate	96	1,415	0.1	4, 2
Methidathion S-[(5-methoxy-2-oxo-1,3,4-thiadiazol-3-(2H)-yl) methyl] O,O-dimethylphosphorodithioate	61	1,384	16.6	4
ALIPHATIC LEAVING GROUP				
Disulfoton O,O-diethyl S-[2-(ethylthio)ethyl] phosphorodithioate	557	1,733	6.0	4, 2
Dichlorvos 2,2-dichloroethenyl dimethyl phosphate	330	1,489	34.6	4
Trichlorfon dimethyl 2,2,2-trichloro-1-hydroxyethyl phosphonate	165	1,339	120.0	4
Phorate O,O-diethyl S-[(ethylthio)methyl] phosphorodithioate	124	1,270	2.6	4
Demeton-S-methyl S-[2-(ethylthio)ethyl] O,O-dimethyl phosphorothioate	96	1,545	260.0	4, 2
Naled 1,2-dibromo-2,3-dichloro-ethyl dimethyl phosphate	94	1,579	603.0	4, 2
Malathion S-[1,2-bis(ethoxycarboxy)ethyl] dimethyl phosphorodithioate	65	1,451	153.0	4, 2
Dimethoate O,O-dimethyl S-[2-(methylamino)-2-oxoethyl] phosphorodithioate	60	1,472	216.0	4, 2
CONTROLS	63	1,416		4, 2

homogenised in 0.5 M NaOH. Porphyrins were extracted from the cultures and examined as their methyl esters by T.L.C. by methods given earlier [4]. Measurements of alkylation rates were carried out by a modification of the method of Fischer and Lohs [10]. 0.05 mmole of organophosphorous compound was dissolved in 0.05 M 4-(*p*-nitrobenzyl) pyridine in ethylene glycol which had been preheated to 80°. Heating was continued at 80°. 0.05 ml aliquots of the solution were removed at 5 min intervals up to 30 min after addition of the compound. The aliquots were added to 0.95 ml of 5% methanolic KOH and the absorbance read at 554 nm. The reaction rate was linear up to 30 min after commencement of the reaction. Assays of uroporphyrinogen decarboxylase were carried out by the method of Elder [11] on cells scraped from 3.5 cm plates 24 hr after incubation with 65 μM paraxon or diazoxon. Pentacarboxylate porphyrin was a gift from Professor Elder.

RESULTS AND DISCUSSION

Table 1 shows the effect of thirty-two organophosphorous compounds of industrial importance on porphyrin accumulation in cultures of chicken embryo hepatocytes. The highest porphyrin concentrations are associated with compounds which cause accumulation of uroporphyrin and heptacarboxylate porphyrin. ALA loading had little effect on the type of porphyrin accumulating with these compounds. With compounds which cause accumulation of coproporphyrin and protoporphyrin, ALA loading caused a marked increase in the relative amount of coproporphyrin produced. Control cultures loaded with ALA produced little porphyrin other than coproporphyrin.

The compounds show a clear division into two groups—those causing coproporphyrin and some protoporphyrin accumulation and those causing uroporphyrin and heptacarboxylate porphyrin accumulation. With few exceptions the most striking structural feature of the latter group is that they are ethyl esters with an aromatic leaving group which is strongly electrophilic. Methyl esters generally are associated with accumulation of coproporphyrin. Since ALA loaded controls also produce coproporphyrin, the most likely explanation for this pattern of porphyrin accumulation is simple induction of the synthesis of cytochrome P-450 and of ALA-synthase, a phenomenon which is well known for lipophilic compounds [12]. The accumulation of coproporphyrin rather than protoporphyrin under these conditions may be characteristic of collagenase treated cells since trypsinised cells accumulate protophorphyrin [13]. Compounds associated with uroporphyrin accumulation may be phosphates (paroxon and diazoxon), phosphorothionates (bromophos-ethyl, pyrimiphosethyl), or phosphorothiolothionates (azinphos-ethyl). The higher activity of the phosphates suggests that this form of the organophosphorous compounds may be the active form. Nonetheless, phosphate methyl esters (fospirate, naled) are inactive as uroporphyrin accumulators. The influence of the ethyl ester on uroporphyrin accumulation is most clearly seen in the compound, chlorpyrifos (3, 5, 6-trichlorophyrid-2-yl diethyl phosphorothionate), a uroporphyrin accumulator, its methyl ester analogue, Dowco 214, a coproporphyrin accumulator, and fospyrate, the phosphate methyl ester analogue which causes little porphyrin accumulation at all. Accumulation of uroporphyrin and heptacarboxylate porphyrin in culture is typical of inhibitors of uroporphyrinogen decarboxylase. Direct assays of cells exposed to paroxon and diazoxon (Table 2) confirm that this is the cause of the accumulation of these porphyrins in cultures treated with these compounds.

The effect of organophosphorous pesticides on enzymic systems is associated with two main types of reactivity—phosphorylation of serine residues of such enzymes as acetylcholine esterase, and alkylation reactions. The rate of alkylation of 4-(*p*-nitrophenyl)-pyridine is a convenient colorimetric measure of alkylation potential. Alkylation rates of this compound are shown in Table 1. Clearly no positive correlation between alkylation rate and porphyrin accumulation exists. In fact, as would be expected, methyl esters show much greater alkylation rates than ethyl esters. Most compounds with good alkylating ability cause little porphyrin accumulation (naled, demeton-*S*-methyl, dichlorvos).

Although hydrolysis rates should provide a convenient guide to phosphorylation potential of organophosphorous compounds, such a study was not undertaken. It is unlikely that the active inhibitor of urophorphyrinogen decarboxylase is a phosphorothionate or phosphorothiolothionate. Such compounds almost certainly undergo oxidative desulphurization to the corresponding phosphates and other metabolites. This is suggested by the marked activity of paroxon and diazoxon which may act as direct inhibitors of the enzyme. Both of these compounds are considerably less lipophilic than their phosphorothionate analogues, indicating that their

Table 2. Inhibition of uroporphyrinogen decarboxylase in cultures of chicken embryo hepatocytes

Coproporphyrin	Activity of uroporphyrinogen decarboxylase (pmole coproporphyrin/min/mg protein)*
Controls	15.32 ± 1.84
Paroxon treated	3.54 ± 0.97†
Diazoxon treated	5.87 ± 1.14†

*Values are means ± S.D. for six determinations.
†$P < 0.005$

effect on porphyrin accumulation is specific and not associated purely with induction of cytochrome P-450 synthesis.

A mechanism which may explain the greater activity of the ethyl esters rests on the proposition that the active inhibitor of uroporphyrinogen decarboxylase is the phosphate analogue of the compound in question.

The main competing reactions in the detoxification of phosphorothioates are oxidative desulphurization to the phosphate and dealkylation reactions, especially glutathione *S*-alkyl transfer. The latter reaction is favoured in the case of methyl phosphorothioates [14]. Ethyl esters are detoxified by the dealkylation routes much less readily. In the case of parathion, for example, experiments with O^{18} indicate that oxidative desulphurization appears to account for most of the breakdown products, paroxon, diethyl phosphorothioate and diethyl phosphate [15]. The reduced rate of detoxification of the ethyl esters, reflected in their increased toxicity to whole animals, may well favour reaction with proteins and peptides other than glutathione. It may also favour reactions involving the aryl leaving group rather than the relatively unreactive alkyl (ethyl) groups. It is reasonable to suggest that this type of reaction may occur between the ethyl organophosphates and the reactive sulphhydryl group of uroporphyrinogen decarboxylase. Indeed, sulphhydryl arylation reactions have been observed with diazoxon in rat liver homogenates by Shishida [16]. This suggestion opens the possibility that organophosphate ethyl esters may prove useful labels for investigation of the reaction of the sulphhydryl group of uroporphyrinogen decarboxylase with intermediates formed during the detoxification of hexachlorobenzene and other organochlorine pesticides [17, 18].

The finding that diazoxon acts as an inhibitor of uroporphyrinogen decarboxylase suggests that some cases of human porphyria cutanea tarda linked to diazinon use may be associated with this metabolite.

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