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Structure-Activity Relationship in Organophosphate-induced β-glucuronidase Release from Rat Hepatocytes *in vitro*

Xin-fu Leng[1] and Tsutomu Nakatsugawa

Department of Environmental and Forest Biology, State University of New York, College of Environmental Science and Forestry, Syracuse, NY 13210

Release of hepatic β-glucuronidase in the rat is one of the most rapid and sensitive responses to exposure to organophosphorus insecticides (WILLIAMS 1969; SUZUKI et al. 1975). The importance of electron-withdrawing effect of phenyl substituents in the dialkyl phenyl ester series of organophosphates in vivo suggests that phosphorylation mechanism underlies the enzyme release (KEADTISUKE & NAKATSUGAWA 1980). The response is, however, apparently distinct from cholinergic effects (STAHL et al. 1975) and results from disturbances of noncholinergic mechanism controlling intracellular enzyme level. Putative target macromolecule may well be located within the hepatocyte because a recent study in our laboratory had demonstrated a dose-dependent release of β-glucuronidase from isolated rat hepatocytes in suspension culture in response to the addition of paraoxon (O, O-diethyl O-4-nitrophenyl phosphate) to the culture medium. While the suspension culture appears to maintain hepatocytes in good health for a relatively short period of a little over an hour, the in vitro response nevertheless appears to be a true reproduction of the effect observed in vivo and the reduced effect of SV1-oxon (O, O-diethyl O-phenyl phosphate) compared with paraoxon also seems to confirm observation in vivo. If hepatocyte suspension is indeed a self-contained model for studying the mechanism of the β-glucuronidase release, it will serve as a useful means of understanding non-cholinergic target macromolecules of organophosphate side effects involving disturbances of intracellular homeostasis. This report presents the result of our survey of organophosphorus esters for their in vitro potency in eliciting the β-glucuronidase release from isolated hepatocytes, confirming the conclusion obtained in earlier experiments in vivo.

MATERIAL AND METHODS

The following analogs of methyl paraoxon were obtained from the Dow Chemical Company, Midland, Michigan. The general structure was O, O-dimethyl O-x-phosphate, where x was phenyl; 4-nitrophenyl; 2,4,5-trichlorophenyl; 2-chloro-4-nitrophenyl; 2,6-di-

[1]Present address: Institute of Zoology, Academia Sinica, Peking, People's Republic of China.

chlorophenyl; 3,4-dichlorophenyl; 4-chlorophenyl; 2-chloro-4-t-butylphenyl; 4-methoxyphenyl; 4-cyanophenyl; 2-chloro-4-cyanophenyl; 2-chlorophenyl; 4-t-butylphenyl; 2,4-dichlorophenyl. The sources of the following compounds were as follows: O, O-diethyl O-4-methylsulfinylphenyl phosphorothioate (Chem Agro Corp., Kansas City M.O.); O, O-diethyl O-4-nitrophenyl phosphorothioate, O, O-diethyl O-2,4-dichlorophenyl phosphorothioate and O, O-diethyl O-2,5-dichloro-4-bromophenyl phosphorothioate (U.S. Environmental Protection Agency. Health Effects Research Lab., Research Triangle Park, N.C.); O, O-diethyl O-4-nitrophenyl phosphorothioate and O, O-dibuyl O-4-nitrophenyl phosphorothioate (American Cyanamid Co. Princeton, N.J.). Following three compounds were prepared in this laboratory (major peaks of respective mass spectrum given in parentheses): O, O-diethyl O-phenyl phosphorothioate (SV_1) (m/e 246 (M^+) (50.29%), 218 (5.11%), 141 (17.02%), 110 (41.34%), 109 (31.91%), 105 (20.79%), 104 (15.33%), 97 (32.25%), 94 (100%), 64 (29.62%); O, O-disopropyl, O-4-nitrophenyl phosphorothioate (m/e 319 (M^+) (11.9%), 277 (20.57%), 235 (100%), 155 (45.07%), 139 (93.26%), 109 (35.37%), 93 (9.8%), 59 (31.54%); O, O-di-n-propyl O-4-nitrophenyl phosphorothioate (m/e 319 (M^+) (2.72%), 277 (13.1%), 236 (100%), 155 (8.28%), 139 (30.83%), 59 (2.27%).

The following materials were purchased from the sources indicated: collagenase (Type I), bovine serum albumin (Fraction V), heparin (Sodium salt, Grade II), phenol red (Sodium salt) (Sigma Chemical Co., St. Louis, M.O.); Waymouth MB 752/1 powder medium, (Grand Island Biological Co., Grand Island, N.Y.).

Rat hepatocytes were isolated by the collagenase perfusion procedure as described before (NAKATSUGAWA et al. 1980) from male Sprague-Dawley strain rats, weighing 280-345g at the time of experiment, purchased from Taconic Farms, Germantown, New York. Hepatocytes were suspended in Waymouth's medium at the concentration of about 2 million cells/ml. and maintained in a spinner flask under carbogen gas (95% O_2, 5% CO_2) at 37^{0}C. The cell viability was 80-100% as measured by a trypan blue exclusion test using a hemacytometer for cell counting. Incubation of hepatocytes with organophosphates was carried out at 37^{0}C in water-jacketed spinner vials (10ml capacity) with conduit for blowing in a stream of carbogen gas and a rubber diaphragm for introducing and withdrawing cell suspensions and solutions. The vials were set on a Multi-Magnester (Lab-line instruments, Inc. Melrose Park, I.L.) and the jacket water was supplied by a circulating water bath (Neslab Instruments, Portsmouth, N.H.). In each vial, 4 ml of hepatocytes suspension and 4 ml of an organophosphate solution were mixed and 0.4 ml aliquots were withdrawn 60 minutes later. Each sample was centrifuged and 0.2 ml of the supernatant was stored at -20^{0}C until enzyme analysis. β-Glucuronidase activity was measured by the fluorometric method

Table 1. β-Glucuronidase release effect and distribution quotient, q, of various organophosphates in hepatocyte suspension.

Organophosphates	β-glucuronidase activity (10^{-6}M 4-MUB in 30 min.)	q value
dimethyl phosphates		
1. 4-nitrophenyl	4.97	55.7
2. 2,4,5,-trichlorophenyl	3.08	46.0
3. 2-chloro-4-nitrophenyl	2.76	8.4
4. 2,6-dichlorophenyl	0.98	8.5
5. 3,4-dichlorophenyl	-0.21	40.0
6. 2,4-dichlorophenyl	0.14	-
7. 4-chlorophenyl	0.87	11.0
8. 4-methoxyphenyl	0.98	7.6
9. 4-t-butylphenyl	1.40	9.2
10. phenyl	0.49	4.7
11. 2-chlorphenyl	0.94	4.1
12. 2-chloro-4-t-butylphenyl	0.63	-
13. 4-cyanophenyl	1.29	54.8
14. 2-chloro-4-cyanophenyl	0.70	2.4
diethyl phosphorothioates		
1. phenyl	0.24	282.9
2. 4-nitrophenyl	7.75	405.2
3. 2,4-dichlorophenyl	1.99	433.0
4. 2,5-dichloro-4-bromophenyl	6.12	462.9
5. 4-methylsulfinylphenyl	4.76	57.5
4-nitrophenyl phosphorothioates		
1. dimethyl	1.61	54.9
2. diethyl	7.75	405.2
3. di-iso-propyl	4.80	199.0
4. di-n-propyl	3.75	476.5
5. di-n-butyl	2.63	283.3

with 4-methylumbelliferyl-β-D-glucuronide as the substrate following the published procedure (KEADTISUKE & NAKATSUGAWA 1980) except incubation was carried out at 37°C. The distribution quotient, q, was determined essentially as reported before (NAKATSUGAWA et al. 1980). A 0.5-ml aliquot of hepatocytes ($2x10^6$cells/ml) in Waymouth's medium was quickly mixed with the equal volume of the medium containing $2x10^{-5}$M organophosphate and 30 seconds later centrifuged to separate supernatant. The organophosphate in 0.4 ml of the supernatant, mixed with 0.1 ml of 0.5M trichloroacetic acid, was extracted with 0.5 ml of hexane

and analyzed by using a Packard 7400 series gas chromatograph equipped with a ^{63}Ni electron-capture detector and a flame photometric detector. A glass column, 120cmx2mm (I.D.) was packed with 70/80 mesh, silylated Chromosorb G coated with 3% Apiezon N.

RESULTS AND DISCUSSION

Potency of various organophosphates was compared for β-glucuronidase release in vitro based on the enzyme release at 60 minutes of incubation when the enzyme titer reached a sufficient level to reflect the potency while the health of hepatocytes had not deteriorated (Table I). To examine structure-activity relationship, ideally the logarithm of the concentration effecting a mid-point release might be used. In the absence of extensive data, titer of the enzyme released above the control in the presence of a constant 10^{-5}M organophosphate was subsituted. This may be justified because the release increases gradually over a wide range (10^{-7} to 10^{-4}M) of concentration in the case of paraoxon and is more directly related to the logarighm of the paraoxon concentration than the paraoxon concentration itself. Thus, the enzyme released (A) and electron-withdrawing parameter (σ^-) showed a substantial correlation ($A=0.47+1.98\sigma^-$, $\gamma=0.70$ Fig. 1A) in the case of 13 dimethyl phenyl phosphates (No. 1-No.13 in Table I) and an excellent correlation for the 5 diethyl phenyl phosphorothioates ($A=0.05+6.32\sigma^-$, $\gamma=0.98$, Fig. 1B).

Earlier studies have indicated supportive influence of lipophilicity of organophosphates in the β-glucuronidase release (SUZUKI et al. 1975, KEADTISUKE & NAKATSUGAWA 1980). Because more lipophilic chemicals are likely to have higher affinities to plasma proteins as well as to hepatocytes, such indications obtained from in vivo studies may reflect greater influence of lipophilicity in favor of partitioning into hepatocytes. In the isolated hepatocyte system used in this work, no proteins were added to the incubation medium and lipophilicity might be expected to directly favor uptake by hepatocytes. Although the logarithm of the distribution quotient, q, may be comparable to π, correlation between log q and π values from the literature (FUJITA et al. 1964) was poor ($\gamma=0.26$) for 10 dimethyl phenyl phosphates (those in Table I except No. 6,9,10,12) whereas it was substantial for the 5 diethyl compounds ($\gamma=0.82$). Thus the in vitro enzyme release was much better correlated with log q ($\gamma=0.47$ and $\gamma=0.65$ for 12 dimethyl series and 5 diethyl compounds respectively) than with π ($\gamma=-0.04$ and $\gamma=0.34$ for 11 dimethyl group, excluding No. 9, 10, 12 and 4 diethyl compounds No. 1-4. Similarly, for the 4-nitrophenyl phosphorothioate homologs, log q showed a reasonable degree of correlation withthe enzyme release potency ($\gamma=0.61$).

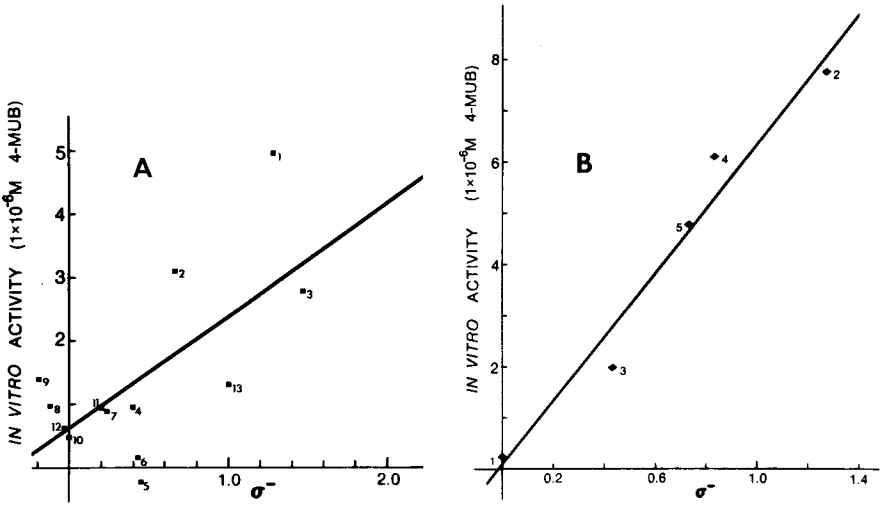

Figure I. Relationship between the in vitro enzyme release and π. (A): For 13 of the dimethyl phenyl phosphates. (B): For 5 diethyl phenyl phosphorothioates. The compound numbers correspond to those in Table I. σ^- values are from SWAIN & LUPTON (1968) and JOHNSON (1973).

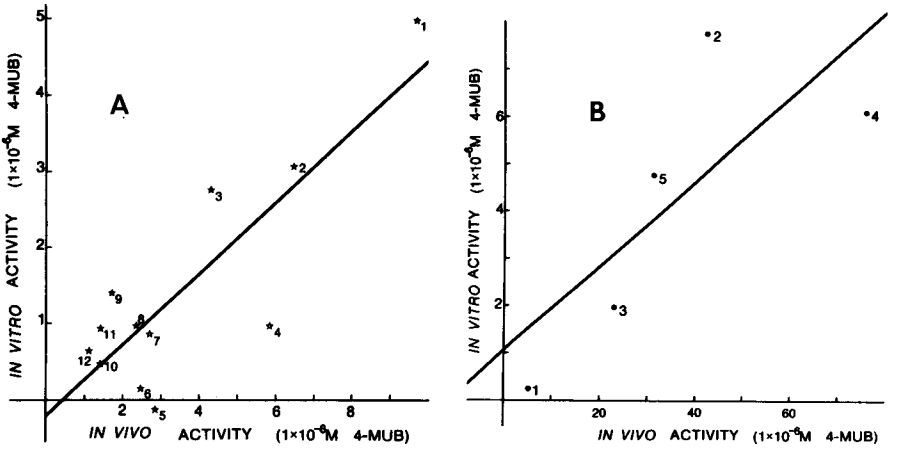

Figure 2. Relationship between the in vitro and in vivo enzyme release. (A): For 12 dimethyl phenyl phosphates. (B): For 5 diethyl phenyl phosphorothioates. The numbers correspond to those in Table 1. Data for enzyme release in vivo are from KEADTISUKE & NAKATSUGAWA (1980).

Part of the generally poor correlations observed among the dimethyl series might reflect their rapid metabolism, which could influence both A and q values even though the phosphorylation generally occurs during the first few minutes (unpublished results) and uptake of organophosphates by hepatocytes is nearly complete within one minute (NAKATSUGAWA et al. 1980). However, the substantial influence of q, i.e. degree of partitioning of organophosphates into the hepatocyte appears to overshadow contribution of π factor, if any, and the potential role of hydrophobicity in the organophosphate-target interaction cannot be assessed at this stage. Interestingly, the enzyme release observed in vitro correlated quite well with in vivo data (Fig. 2A & B). Thus, the regression for the dimethyl phenyl phosphates was A in vitro =-0.24+0.47 A in vivo, (γ=0.83), where as that for diethyl phenyl phosphorothioates, was A in vitro = 1.01+0.089 A in vivo, (γ=0.77). While more detailed data are needed to evaluate the contribution of various parameters definitively, these results are consistent with the hypothesis that phosphorylation mechanism underlies the β-glucuronidase release and in addition provide further evidence that isolated hepatocytes are valid, self-contained, in vitro model for further studies of organophosphate-induced β-glucuronidase release.

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