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Metabolism of Phosphoric Acid Triesters by Rat Liver Homogenate

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In the previous paper (Sasaki et al. 1981), we reported that the absorption and elimination rates of various phosphoric acid triesters (phosphates) in fish vary widely, indicating that the metabolic ability of fish to transform the phosphates is diverse. Consequently, bioaccumulation of such phosphates in fish will depend markedly on the chemical structures.

Very little work has been done on the metabolism of phosphates in fish, and only a few reports are available on the metabolism in mammals; namely Nomeir et al. (1981) investigated the metabolism of tris(1,3-dichloroisopropyl) phosphate (TDCPP) by rats and Suzuki et al. (1984) reported the identification of tributylphosphate (TBP) metabolites in rat urine. Thus, in order to obtain basic data on the metabolic transformations of phosphates, the decomposition of 4 phosphates *in vitro* by a rat liver preparation was examined and the effects of coenzymes and inhibitors on the metabolism were investigated to elucidate the predominant enzyme reaction involved.

MATERIALS AND METHODS

Tributylphosphate (TBP) and diphenyl hydrogen phosphate (DPP) were purchased from Wako Pure Chem. Co., and tris(2-chloroethyl) phosphate (TCEP), tris(1,3-dichloroisopropyl) phosphate (TDCPP), triphenyl phosphate (TPP) and dibutyl hydrogen phosphate (DBP) were purchased from Tokyo Kasei Industry Co. Dibutyl 3-hydroxybutyl phosphate (TBP-OH) and butyl bis(3-hydroxybutyl) phosphate [TBP-$(OH)_2$] were synthesized and purified as described previously (Suzuki et al. 1984). Bis(1,3-dichloroisopropyl) hydrogen phosphate was synthesized from 1,3-dichloroisopropanol and phosphorus oxychloride. Solvents were distilled before use in an all-glass Widmer distillation unit.

0.1 M potassium phosphate (pH 7.4) or 0.1 M Tris-HCl (pH 8.3 or 8.6) buffer was used. For the examination of optimum pH, 0.1 M potassium phosphate (pH 5.8-7.8), 0.1 M Tris-HCl (pH 8.3-8.7) and glycine-NaOH (pH 8.9-10.8) buffers were used.

Ethanol solutions of phosphate (4×10^{-4} mol/l) were used as substrate. Male Wistar rats weighing 150-200 g were used.

Rats were decapitated and the livers were removed and homogenized with 3 volumes of 1.15 % KCl solution. Microsomes and soluble fraction were isolated by centrifugation of the 9000 g supernatant at 105000 g for 60 min. The soluble fraction was dialyzed for 24 hours at 5°C against several changes of 50 volumes of 1.15 % KCl solution.

For the determination of the decrease of substrate as one of the indices of metabolic activity, a typical incubation mixture (final volume of 5-6 ml) consisted of microsomal suspension or soluble fraction, substrate solution (20-50 µl), 4 ml of buffer solution and, if necessary, β-$NADP^+$(1 µmol), glucose-6-phosphate (10 µmol) and glucose-6-phosphate dehydrogenase (2.8 units) or an inhibitor such as SKF-525A. Except in the case of time course experiments, the mixtures were incubated for 30 minutes at 37°C. After the incubation, the reaction mixture was cooled to 0°C and extracted with 5 ml of hexane or ethyl acetate, and the extract was subjected to FPD gas chromatography. The activity of the enzymes decomposing the phosphates was expressed with reference to the decrease in the amounts of substrates during the reactions.
For the precise chemical identification of metabolites, mono- or di-esters of phosphoric acid, TBP-OH and TBP-$(OH)_2$, the reactions were terminated by the addition of 2 ml of 2N HCl, and the mixtures were extracted with 6 ml of ethyl acetate. The acidic metabolites were methylated with ethereal diazomethane prior to FPD-GC analysis.

In order to accumulate sufficient material for the chemical characterization of TBP metabolites, 9000 g supernatant (0.4 ml) of the liver from rats induced by phenobarbital administration was incubated with TBP (400 µg) in pH 7.4 phosphate buffer (15 ml) for 2.5 hr. The reaction mixtures were combined (total 2.5 l) and extracted with hexane. The extract was concentrated and the residue was subjected to silica gel column chromatography to separate TBP. The eluate was acetylated with acetic anhydride and pyridine.

The methods of GLC determination were described in detail in the previous report (Sasaki et al. 1981); the column temperature was set at 150 to 290°C depending on the phosphate to be analyzed.

RESULTS AND DISCUSSION

Rat liver microsomes were observed to possess enzymes decomposing TBP, TDCPP and TPP in the presence of NADPH (Table 1). A study on the time course of the microsomal metabolism (Fig. 1) indicated that TBP was most rapidly decomposed, followed by TPP, then TDCPP. In the absence of NADPH, only TPP was easily decomposed (66 %), whereas TBP (11 %) and TDCPP (7 %) were not. This result shows that arylesterase in the microsomes contributes to TPP metabolism, as discussed later.

Table 1. Metabolism of various phosphates by rat liver microsomes and soluble fraction

compound	extent of reaction (%) microsomes[1]		soluble fraction[2]	
	control	+ $NADP^+$	control	+ SKF-525A
TBP	11	100	22	0
TCEP	19	15	1	-
TDCPP	7	43	36	-
TPP	66	91	15	-

The reaction mixture was incubated at 37°C for 30 minutes in pH 7.4 phosphate buffer.
1) equivalent to 1000 mg of liver
2) equivalent to 350 mg of liver

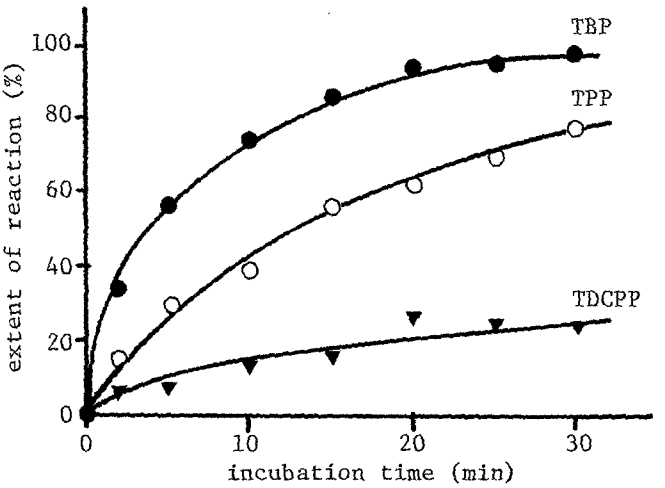


Figure 1. Time course of metabolism of phosphates by rat liver microsomes.
Reaction conditions were as described in Table 2 except that the amount of microsomes was decreased (equivalent to 20 mg of liver).

The soluble fraction metabolized 15-36 % of substrates other than TCEP.
The decomposition of TBP by the soluble fraction probably resulted from microsomal contamination of the soluble fraction, since the reaction was inhibited by SKF-525A, an inhibitor of hepatic mixed function oxidase (MFO) activity (Table 1). In the case of TDCPP and TPP, the inhibitory effect of SKF-525A could not be examined, because SKF-525A reacted directly with these two substrates in the buffer solution without microsomes. However, TDCPP might be metabolized by some enzymes in the soluble fraction, since it was decomposed more than TBP, whereas the reverse would be expected if contaminating MFO were responsible for the decompositions (Table 1). Microsomes and soluble fraction both had little activity for TCEP decomposition under the present experimental conditions.

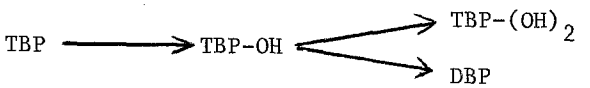
Table 2. Inhibition of the rat liver microsomal metabolism of phosphates

treatment	extent of reaction (%)[1] TBP	TDCPP	TPP
control[2]	100	100	100
- $NADP^+$	10	35	20
+ SKF-525A[3] 0.5 mM	5	17	12
+ KCN[3] 1.0 mM	93	96	96
+ NaN_3[3] 1.0 mM	99	65	86
+ α,α-dipyridyl[3] 1.0 mM	70	78	74
+ EDTA[3] 0.5 mM	101	139	108
+ carbon monoxide	16	17	18

1) Values represent extent of reaction (control = 100).
2) The control reaction mixture consisted of 2 ml of microsomal solution (equivalent to 32 mg of liver), $MgSO_4$ 10 μmol, 0.5 ml of NADPH regenerating system ($NADP^+$ 1 μmol), 2.2 ml of 0.1 M phosphate buffer (pH 7.4) and substrate (ethanol solution) 8 nmol, and was incubated at 37°C for 15 min.
3) Inhibitor was added simultaneously with substrate.

Data presented in Table 2 demonstrate the inhibitory effects of some chemicals and carbon monoxide on the metabolism of phosphates by the microsomes. The metabolic reactions were inhibited almost completely by SKF-525A and carbon monoxide and in the absence of NADPH, whereas KCN, NaN_3, α,α-dipyridyl and EDTA showed little effect. It was concluded from these results that MFO in microsomes plays a central role in the metabolism of these phosphates.

TBP was metabolized by microsomes (corresponding to 32 mg of liver) almost exclusively to one metabolite which was extractable from neutral solution by hexane and contained phosphorus. The metabolite was identified as TBP-OH by GC-mass, IR and NMR spectral analyses of the acetyl ester of the metabolite in comparison with an authentic specimen. The spectral data for the authentic specimen were presented previously (Suzuki et al. 1984).
However, metabolism of TBP with 9000 g supernatant during prolonged incubation yielded two metabolites, $TBP\text{-}(OH)_2$ and DBP, in addition to TBP-OH (Fig. 2).
From the results of the present *in vitro* study, the metabolic pathway of TBP appears to be as follows.



Recently, we reported that the main metabolites in urine of rats given TBP or TBP-OH or dibutyl 3-oxobutyl phosphate were DBP, butyl dihydrogen phosphate and a little $TBP\text{-}(OH)_2$, and furthermore we showed that the hydroxylation of the butyl moiety was catalyzed by MFO, while glutathione S-transferase was concerned in the

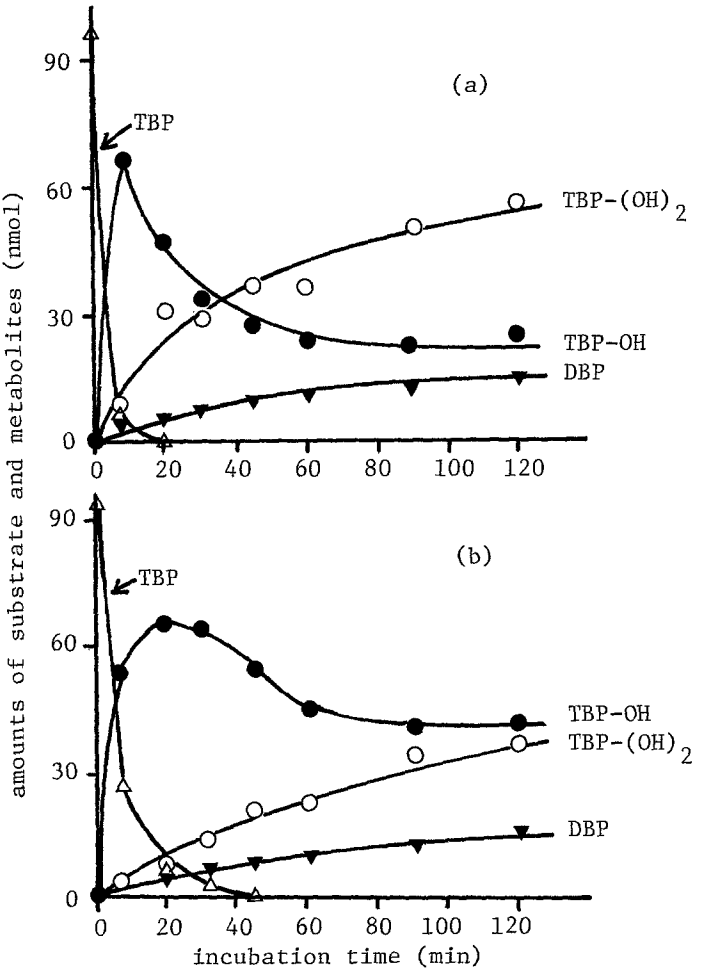


Figure 2. Metabolism of TBP by rat liver 9000 g supernatant. (a): equivalent to 250 mg of liver, (b): equivalent to 125 mg of liver

release of the butyl or hydroxybutyl moiety from phosphate (Suzuki et al. 1984).
However, in the present *in vitro* study, TBP-$(OH)_2$ was formed in a larger amount than DBP from either TBP or TBP-OH (Table 3). The amount of DBP obtained did not increase even when NAD and GSH were added to the incubation medium in order to activate alcohol dehydrogenase and glutathione S-transferase, respectively. DBP was not decomposed at all by the rat liver homogenate, and this result is comparable to that after intraperitoneal administration of DBP (Suzuki et al. 1984).

As described previously, though TDCPP was metabolized by microsomal MFO with an optimum pH of 7.4, it was also metabolized (unlike other phosphates) by enzymes contained in the soluble fraction with a broad optimum pH range, as shown in Fig. 3.

Table 3. Metabolism of TBP, TBP-OH and DBP by rat liver homogenate

substrate	cofactors	substrates and metabolites[1] TBP	TBP-OH	$TBP-(OH)_2$	DBP
TBP	NADP	25	42	6	3
	NADP, NAD	1	38	13	7
	NADP, NAD, GSH	0	35	14	8
TBP-OH	NADP	0	44	33	13
	NADP, NAD	0	16	49	19
	NADP, NAD, GSH	0	13	51	18
DBP	NADP	-	-	-	100

1) Values represent percentages of applied substrates.
The incubation mixture consisted of 2 ml of liver homogenate (700 g supernatant, equivalent to 500 mg of liver), 0.5 ml of NADPH generating system ($NADP^+$ 1 μmol), NAD 1 μmol, GSH 5 μmol, substrate (ethanol solution) 75 nmol and 10 ml of Tris-HCl buffer (pH 8.3).

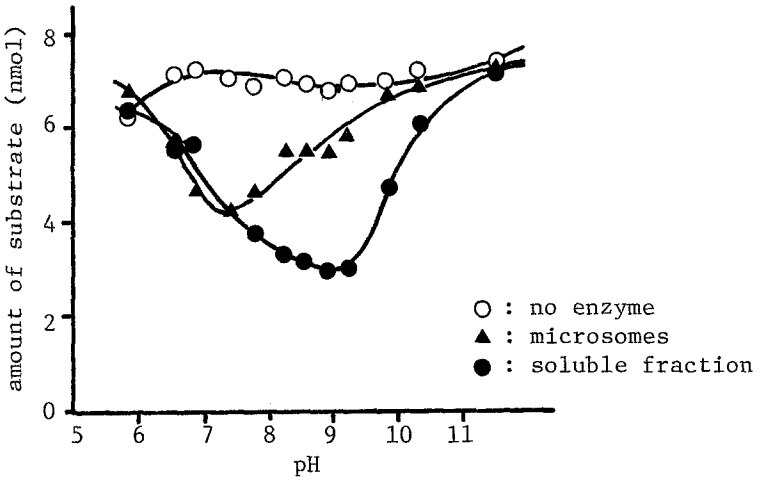


Figure 3. The effect of pH on the metabolism of TDCPP by rat liver microsomes and soluble fraction.
The incubation mixture consisted of 1 ml of microsome fraction (equivalent to 100 mg of liver) or soluble fraction (equivalent to 350 mg of liver), 4 ml of 0.1 M buffer and substrate (ethanol solution) 8 nmol. The microsomal fraction was supplemented with 0.5 ml of NADPH regenerating system ($NADP^+$ 1 μmol).

Microsomal MFO metabolized TDCPP to bis(1,3-dichloroisopropyl) hydrogen phosphate in a yield corresponding to 75 % of the decreased substrate.
On the other hand, Table 4 shows that the dialyzed soluble fraction was not capable of decomposing TDCPP in the absence of GSH. Furthermore, the soluble fraction lost its ability to metabolize TDCPP upon addition of 2,4-dinitrochlorobenzene, an inhibitor of glutathione S-transferase, but the activity was largely recovered after addition of GSH (Table 5). These results indicate that glutathione S-transferase in the soluble fraction is

Table 4. Metabolism of TDCPP by the rat liver soluble fraction

treatment	extent of reaction (%) A[1]	B[2]
control	17.5	13.4
dialyzed	0.4	0
dialyzed and GSH[3] added	18.6	14.4

1) soluble fraction equivalent to 125 mg of liver
2) soluble fraction equivalent to 75 mg of liver
3) 1×10^{-3} M

Table 5. Inhibitory effect of 2,4-dinitrochlorobenzene (2,4-DNCB) on the metabolism of TDCPP by the rat liver soluble fraction

composition of reaction mixture preincubation[1]	reaction[2]	extent of reaction (%)
sup[3]	+ TDCPP	21.4
sup[3]	+ TDCPP, GSH[4]	27.6
sup[3], GSH4)	+ TDCPP	30.6
sup[3], 2,4-DNCB[5]	+ TDCPP	0
sup[3], 2,4-DNCB[5]	+ TDCPP, GSH[4]	13.7
1.15 % KCl, GSH[4]	+ TDCPP	0
1.15 % KCl, 2,4-DNCB[5]	+ TDCPP	0
1.15 % KCl, 2,4-DNCB[5], GSH[4]	+ TDCPP	0

1) at 37°C for 10 min.
2) at 37°C for 20 min.
3) soluble fraction equivalent to 100 mg of liver
4) 1×10^{-3} M
5) 2×10^{-4} M

a major contributor to the TDCPP metabolism.
Since no metabolites were extracted by organic solvent from the soluble fraction incubation mixture, it appears that TDCPP is directly conjugated with glutathione as reported by Nomeir et al. (1981).
TDCPP metabolism in the soluble fraction decreased to 40 - 50 % in the presence of microsomes (data not shown). A similar phenomenon has been observed in the GSH-dependent metabolism of diazinon (Shishido et al. 1978).

TPP was metabolized by microsomes without NADPH, unlike other phosphates (Table 1), and this reaction had a broad range of optimum pH (7-9).
Table 6 shows the decomposition of phosphate at pH 8.6 by the microsomes; at this pH, MFO should contribute little to the metabolism of TPP, since even TBP, which is susceptible to MFO at pH 7.4, was only slightly decomposed.
DPP was the only major metabolite of TPP produced by both MFO and this enzyme, and DPP was not decomposed by microsomes. From these results, arylesterase was supposed to be involved in this metabolic

Table 6. Metabolism of phosphates by rat liver microsomes without NADPH at pH 8.6

substrate	extent of reaction (%)
TBP	11
TDCPP	13
TPP	69

The reaction mixture contained microsomes equivalent to 1000 mg of liver and substrate in Tris-HCl buffer solution (pH 8.6).

transformation, but further investigation was not carried out because the enzyme activity was weak.

The metabolic decomposition of 4 phosphates by a rat liver preparation *in vitro* was investigated with the following results.

1) TBP was easily oxidized to TBP-OH and further to TBP-$(OH)_2$ by microsomal MFO.

2) TDCPP was decomposed partly by MFO to bis(1,3-dichloroisopropyl) phosphate, and was metabolized more efficiently to the glutathione conjugate by the soluble fraction.

3) TPP was decomposed to DPP by both MFO and arylesterase in microsomes.

4) TCEP was scarcely metabolized by rat liver.

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