Studies on the Effects of Phthalate Esters on the Biological System (Part 2)—*In Vitro* Metabolism and Biliary Excretion of Phthalate Esters in Rats

Hiroyasu Kaneshima[1], Tomohiro Yamaguchi[2], Toyo Okui[1] and Mie Naitoh[1]

[1]*Hokkaido Institute of Public Health N-19, W-12, Kitaku, Sapporo, 060, Japan*

[2]*Laboratory of Health Education Faculty of Education Hokkaido University N-11, W-7, Kitaku, Sapporo, 060, Japan*

Phthalate esters (PAE) used extensively as plasticizers are known to have widely contaminated our environment (Mayer 1972, Williams 1973, and Ohta and Katase 1975). However, the toxicities and metabolism of PAE in mammals have not been cleared up enough (Williams and Blanchfield 1974). The previous studies (Yamaguchi et al. 1976) on the distribution of ^{14}C-dibutyl phthalate (^{14}C-DBP) in mice by whole body autoradiography indicated that orally administered ^{14}C-DBP to male mice was readily absorbed from the gastrointestinal tract and accumulated mainly in the liver and kidneys during 6 hr after administration. Therefore, it seems to be of interest to study the abilities of liver and kidneys to metabolize PAE and biliary excretion related to the metabolic fate of them.

The present paper describes the in vitro metabolism of PAE with the liver and kidney homogenates of rat, the biliary excretion in rat after administration of ^{14}C-DBP, and the identification of its metabolites in the bile.

Materials and Methods

Materials

Dimethyl phthalate (DMP), di-n-butyl phthalate (DBP), diethylhexyl phthalate (DEHP) and di-n-octyl phthalate (DOP) were obtained from Tokyo Kasei Co. and DBP labelled with ^{14}C in the carboxyl group was prepared as described previously (specific activity; 0.59 μCi/mg). Labelled DBP was diluted with non-radioactive DBP to the proper specific activity according to the planned experiment. Other chemicals were of reagent grade and used without further purification.

In Vitro Metabolism

Male Wistar albino rats weighing about 250 g were sacrificed after fasting for 12 hr,and liver and kidneys were removed rapidly. Then, 20 % both tissue homogenates were prepared in ice-cold physiological saline containing 1 % D-glucose (pH 6.5) employing a potter glass homogenizer separately. 5 ml of liver or kidney homogenates were transferred to 5 test tubes respectively, containing

Bulletin of Environmental Contamination & Toxicology,
007-4861/78/0019-0502 $1.60

2 mg of each PAE (DMP, DBP,DEHP and DOP) dissolved in 0.2 ml of 50 % ethanol.

The mixture was incubated at 37° under air for various periods of time. At the end of the incubation, the mixture was acidified to pH 2-3 with 4 N hydrochloric acid and extracted three times with 10 ml of n-hexane. The combined extracts were concentrated at 40° under reduced pressure and the residues were dissolved in 1 ml of n-hexane for the determination by gas chromatography (GLC).

Gas Chromatography

PAE in tissue extracts were determined by using a Shimadzu gas chromatograph GC-6A equipped with a flame ionization detector. The column used was a 3 mm i.d. glass, 2 m in length, packed with 2 % OV-1 on 80-100 mesh Chromosorb W treated with AW-DMCS. The column and detector temperatures were 140-250° (6°/min) and 260° respectively. The concentration of each PAE was calculated from the standard calibration curve.

Biliary Excretion

The male rats weighing about 300 g were fasted for 15 hr before oral administration and then given ^{14}C-DBP of 5 μCi dissolved in 50 % ethanol in a single dose 500 mg/kg through a gastric tube. Under ether anesthesia, the common bile duct was exposed by a midline abdominal incision and cannulated with polyethylene tube. The bile was collected periodically every 1 hr for 6 hr period. Each 100 μl aliquot was assayed for radioactivity.

Radioactivity Measurement

The radioactivity in bile was directly measured by a Beckman LS-230 liquid scintillation spectrometer with an automatic quenching monitor, using a counting medium which consisted of 4 g PPO, 50 mg POPOP and 1000 ml of dioxane.

Identification of DBP and Its Metabolites in Bile

In order to study the metabolites, an aliquot of the pooled bile was extracted with ether under acidic conditions. The ether extracts were evaporated at 40° under reduced pressure and the residue was redissolved in 0.2 ml of ether for the separation by thin-layer chromatography (TLC). For the study of conjugated metabolites, the aqueous phase remaining after ether extraction was adjusted to pH 4.5 (acetate buffer), treated with β-glucuronidase for 15 hr at 37°, and the resulting deconjugated materials were extracted with ether and chromatographed as described for the free metabolites.

Thin-layer Chromatography and Formation of Derivatives for Gas Chromatography

DBP and its metabolites were separated using a thin-layer plate (20 × 20 cm, 250 mm thickness) of Kieselgel HF_{254} (Merck). The TLC plate was developed using the solvent system chloroform-methanol-acetic acid, 143:7:1.5, v/v (Albro et al. 1974a) and ethanol-conc.NH_4OH-water, 150:18:24, v/v. Pure standards were run simultaneously on the same plate. The separated spots were detected with ultraviolet light, scraped off, and extracted with ethylacetate. The combined extracts were evaporated under reduced pressure and the residue was dissolved in 0.1 ml of pyridine and 40 μl of N,O-bis(trimethylsilyl)-acetamide, BSA. The reaction time was 1 hr at 50°. GLC of trimethylsilyl (TMS) derivatives was carried out following the procedure described above except the column temperature used was 190°.

Results and Discussion

In Vitro Metabolism of PAE

Spontaneous degradation of PAE only in physiological saline containing 1 % D-glucose at 37° was negligible.

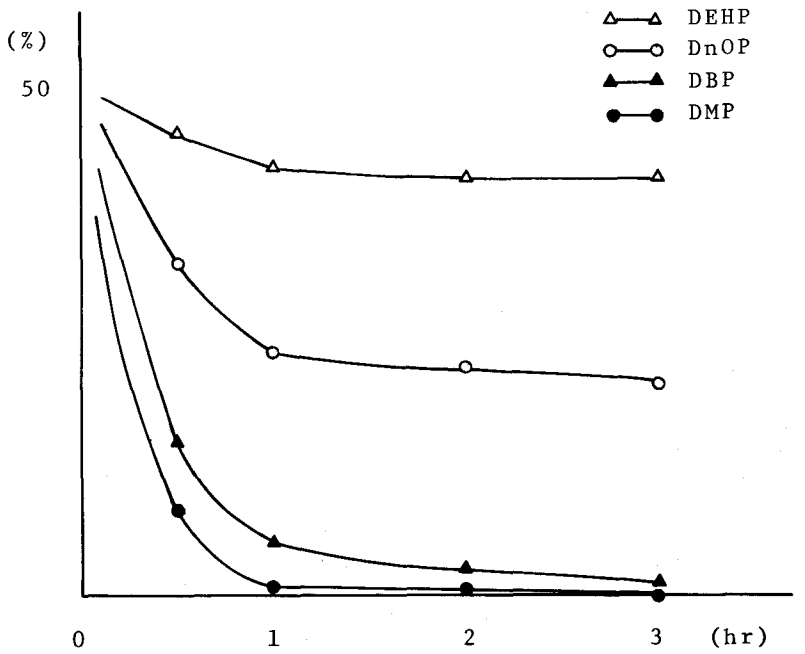

Fig.1 Degradation curves of PAE by liver homogenates of rats

However, when incubated with rat liver homogenates at 37°, added PAE was degraded at various rates as shown in Fig.1. DEHP and DOP were gradually degraded with time by the rat liver homogenates, while DMP and DBP were rapidly degraded and almost disappeared 2 hr after incubation. The metabolic rate of DOP was also faster than that of DEHP having alkyl side chain. The results revealed that PAE having lower molecular weight were metabolized at a faster rate in rat liver and the difference in the metabolic rates was due to the difference in their chemical structures.

As shown in Fig.2, the rate of degradation of PAE by rat kidney homogenates was relatively slow compared with that by rat liver homogenates, however, 70-95 % of PAE initially added were degraded at 5 hr after incubation.

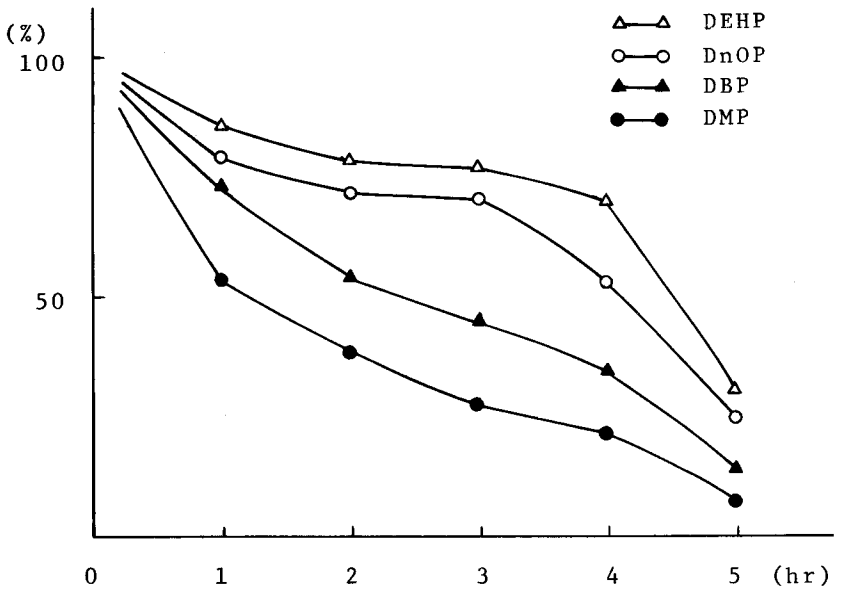

Fig.2 Degradation curves of PAE by kidney homogenates of rats

The similar tendency in metabolic pattern was observed in rat kidneys and DMP having the lowest molecular weight of PAE used was degraded with the fastest rate. Thus, these findings demonstrate that there is a direct relationship between the molecular weight or chemical structures of PAE and the metabolic rate of them in the liver and kidneys of rats.

From the in vitro metabolic studies it was shown that the liver and kidneys of rats have the ability to metabolize PAE

rapidly because PAE were found not to be degraded spontaneously under these experimental conditions. Since PAE were found to be metabolized rapidly by the liver and kidneys of rats, it appears to be of value to investigate whether the metabolites of PAE produce any harmful or toxic effects in living systems.

Biliary Excretion of ^{14}C-DBP

The excretion of radioactivity in the bile after intravenous or oral administration of ^{14}C-DBP to male rats is shown in Fig.3. A relatively rapid excretion of the radioactivity was observed soon after the intravenous injection of ^{14}C-DBP, recovering about 10 % of the dose during 5 hr, while only 4.5 % of the radioactivity was recovered during 6 hr after oral administration of ^{14}C-DBP. This result may indicate that the excretion of DBP through the biliary route is evidenced to have some role in its metabolic fate.

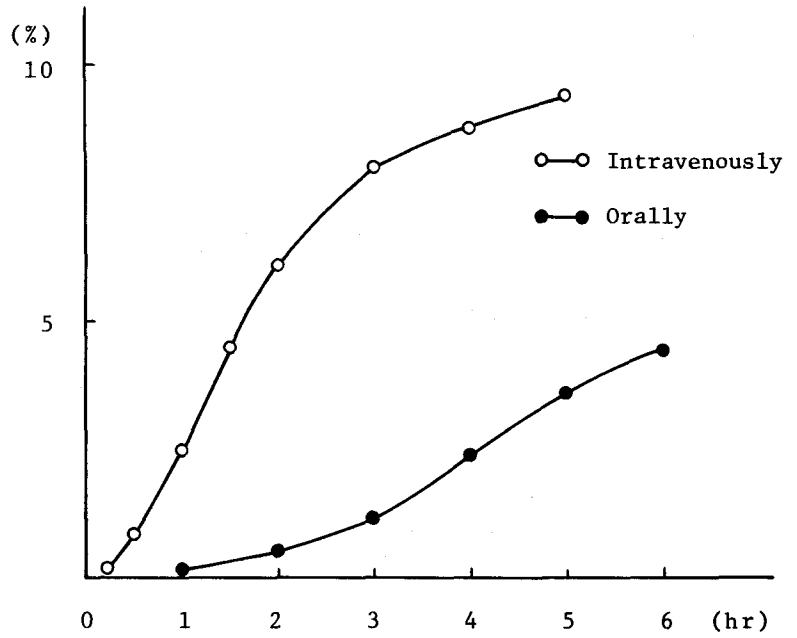

Fig.3 Cumulative biliary excretion of radioactivity in rats after oral or intravenous administration of ^{14}C-DBP

As reported previously, DBP absorbed from rat intestines was slowly excreted in the feces, therefore, it could be considered

that some part of DBP absorbed was brought back into the intestines through the biliary excretion, then excreted in the feces.

Metabolites in the Bile

Table I demonstrates the result of TLC of the ether extract from rat bile. Five or four distinctive spots were detected on thin-layer developed in chloroform-methanol-acetic acid (143:7:1.5) or in ethanol-conc.NH_4OH-water (150:18:24), respectively and three of them were identified as DBP, monobutyl phthalate (MBP), and phthalic acid by comparison of R_f values of reference standards.

TABLE I

R_f Values of Metabolites and References

Solvent*	R_f Value			
	DBP	MBP	PHA**	Bile Spots
I	0.76	0.30	0	0.77,0.58,0.30 0.13,0
II	0.91	0.82	0.26	0.91,0.80,0.61 0.24

* : I chloroform-methanol-acetic acid (143:7:1.5)

II ethanol-conc.NH_4OH-water (150:18:24)

** : PHA. Phthalic acid

Further, these compounds were confirmed by GLC on 2 % OV-1 and DBP was identified by direct comparison with reference standard but MBP and phthalic acid were identified as TMS derivatives as shown in Fig.4.

On the other hand, GLC analysis of the ether extracts from the aqueous phase indicated that the bile contained a glucuronide of MBP and traces of other glucuronides as shown in Fig.5. Similar results were obtained by Okada and Tamemasa (1976) who reported that free MBP and a glucuronide of MBP were detected as the main metabolite and conjugate, respectively, in the liver of mice after

oral administration of ^{14}C-DBP.

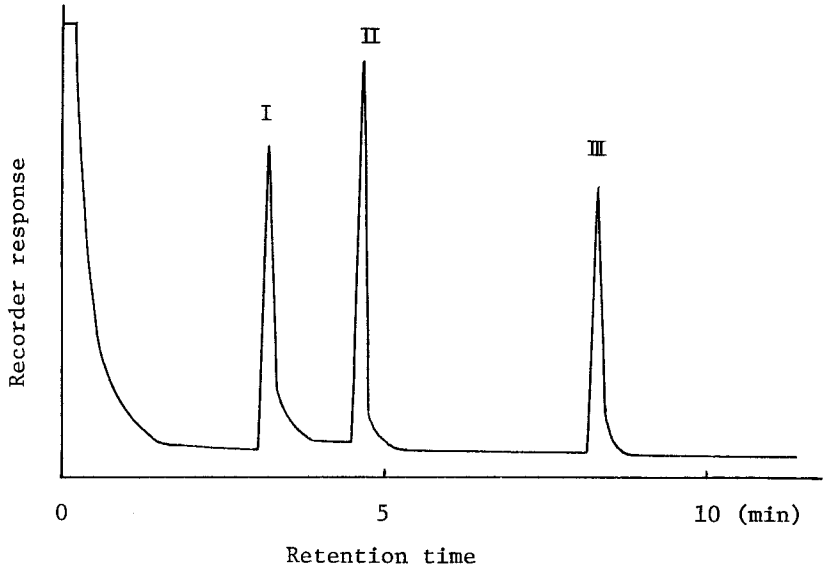

Fig.4 Gas chromatogram of DBP and TMS derivatives of MBP and phthalic acid (PHA)
I : PHA-TMS II : MBP-TMS III : DBP

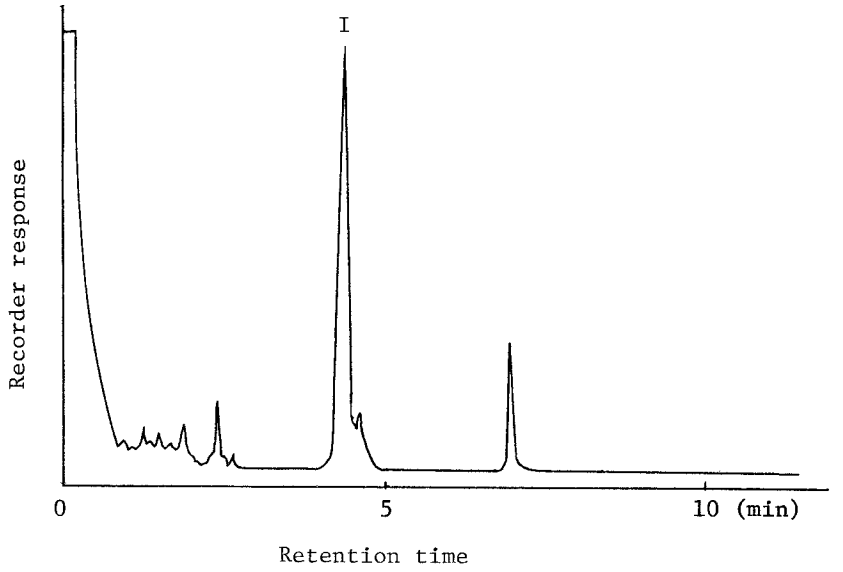

Fig.5 Gas chromatogram of the ether extract from the aqueous phase after treatment of β-glucuronidase
I : MBP-TMS

Albro and Thomas (1973b) reported that pancreas homogenate was able to hydrolyze DEHP to mono-(2-ethylhexyl) phthalate and suggested that orally ingested DEHP would have little opportunity to be absorbed intact. However, the fact that orally given DBP was found in the bile may indicate that a small part of DBP is absorbed through the intestines as the unaltered form.

Further studies on the identification of unknown spots on thin-layer are required for an elucidation of the metabolic fate of PAE in mammals.

Acknowledgments

We are indebted to Mr.Hiroshi Ogawa for the preparation of the labelled compound.

References

ALBRO, P. W. et al. : J. Chromatog. 76, 321 (1973a).

ALBRO, P. W. and THOMAS,R. O. : Biochim. Biophys. Acta 360, 380 (1973b).

MAYER, F. L. Jr. et al. : Nature 238, 411 (1972).

OHTA, H. and KATASE, T. : Kagaku 45, No.3, 182 (1975).

OKADA, S. and TAMEMASA, O. : Abstracts of Papers, The 96th Annual Meeting of Pharmaceutical Society of Japan, Nagoya, April, 1976, p. 167.

WILLIAMS, D.T. : J. Ass. Off. Anal. Chem. 56, 181 (1973).

WILLIAMS, D.T. and BLANCHFIELD, B.J. : Bull. Environ. Contaminat. and Toxicol. 11, 371 (1974).

YAMAGUCHI, T. et al. : Jap. J. Hyg. 31, 331 (1976).