Effect of Phthalate Ester Metabolites on Rat Liver *

A. E. GANNING,^a E. KLASSON,^a
Å. BERGMAN,^a U. BRUNK^b and
G. DALLNER^c

^a Department of Biochemistry and Wallenberg Laboratory, University of Stockholm, Stockholm,

^b Department of Pathology, University of Linköping, Linköping and ^c Department of Pathology, Karolinska Institutet, Stockholm, Sweden

Plasticizers are used extensively in industrial processes, and a relatively high level of contamination is found in food and drinking water.¹ Measurements of the air in cities and analysis of earth also detected a variable content of phthalate esters.² In previous investigations we have found prominent induction of peroxisomes and mitochondria in liver of rats obtaining phthalate ester in their diet.³ Judging from the metabolite pattern excreted from the urine, it is obvious that an extensive metabolism of phthalate esters take place before they are eliminated as water soluble compounds. Consequently, it is very possible that it is not the substrate but some of the metabolites which exert their effects on the liver. For this reason, we have synthesized some of the main known metabolites of di(2-ethylhexyl)phthalate ester (DEHP), which were subsequently fed to rats.

The compounds tested were DEHP, mono-(2-ethylhexyl)phthalate ester (MEHP), 2-ethylhexylbenzoate (EHB), phthalic acid (PA) and 2-ethylhexanol (EH). Rats were fed *ad libitum* with diet containing 2% of the above compounds for two weeks. At the end of this period the liver was

removed, pieces were taken for electron microscopy, and the remainder was homogenized and subfractionated to obtain mitochondria and microsomes. Protein and various enzyme activities were measured as described earlier.³

The DEHP concentration of 2% in the diet was chosen because this concentration has been shown to give maximal induction within 2 weeks according to previous investigations.⁴ Experiments were also performed using diets containing 0.2 and 0.02% DEHP during a 6-month period and the pattern of morphological, chemical and enzymatic changes were very similar to those described below with 2% DEHP. As can be expected, however, the magnitude of the changes was lower.

Induction of peroxisomes can be followed by measuring enzymes known to be localized exclusively in these particles. The mitochondrial β -oxidation of fatty acids is dependent on the respiratory chain and consequently is highly sensitive to KCN. The respiratory chain is not present in peroxisomes, which is the only location for extramitochondrial β -oxidation of fatty acids. This makes it possible to follow the peroxisomal β -oxidation enzymes in the homogenate.⁵ As is established in previous experiments, DEHP induces substantially palmitoyl-CoA oxidation (Table 1). MEHP, which is the main metabolite of the ingested substrate, exerts a similar effect. Interestingly, two major metabolites, PA and EH, do not display an effect on peroxisomal β -oxidation. EHB which differs from MEHP by the lack of the carboxyl group is completely ineffective for induction of peroxisomes and peroxisomal enzymes. The specific activity of catalase and urate oxidase are decreased after administration of DEHP whereas the various metabolites tested produced either no change or gave a slight increase in the activities.

One of the unusual effects of DEHP on liver is the pronounced effect on mitochondria which are induced fourfold in the initial two weeks period. The induction involves an increase in the number of mitochondria without changes in the specific

* Communication at the Meeting of the Swedish Biochemical Society in Stockholm, 26–27th November, 1981.

Table 1. Effect of DEHP and various metabolites on peroxisomal enzymes in homogenates.

Treatment	Palmitoyl-CoA oxidation (nmol NAD reduced) $\text{min}^{-1} (\text{mg protein})^{-1}$	Catalase ($\mu\text{mol H}_2\text{O}_2$ decomposed) $\text{min}^{-1} (\text{mg protein})^{-1}$	Urate oxidase (nmol urate oxidized) $\text{min}^{-1} (\text{mg protein})^{-1}$
Control	4.8	81	37
DEHP	28.8	49	23
MEHP	16.7	113	35
EHB	3.5	65	30
PA	4.2	70	51
EH	3.8	81	40

Table 2. Effect of DEHP and various metabolites on mitochondrial enzymes measured in isolated mitochondria.

Treatment	Protein mg/g liver	Cytochrome <i>c</i> oxidase ($\mu\text{mol cyt } c \text{ ox}$) $\text{min}^{-1} (\text{mg protein})^{-1}$	Carnitine-acetyl transferase (nmol CoA produced) $\text{min}^{-1} (\text{mg protein})^{-1}$
Control	18.1	1.2	0.75
DEHP	49.3	1.1	21.8
MEHP	43.0	2.3	7.7
EHB	18.9	0.9	0.7
PA	19.5	2.1	0.8
EH	17.3	1.3	0.9

Table 3. Effect of DEHP and various metabolites on microsomal enzymes measured in isolated microsomes.

Treatment	Protein mg/g liver	Cytochrome P-450 nmol (mg protein) $^{-1}$	NADPH-cytochrome <i>c</i> reductase ($\mu\text{mol cyt } c \text{ red}$) $\text{min}^{-1} (\text{mg protein})^{-1}$
Control	18.6	0.60	0.089
DEHP	19.2	0.98	0.142
MEHP	18.6	1.01	0.154
EHB	17.1	0.67	0.092
PA	18.0	0.58	0.095
EH	19.8	0.68	0.101

activities of enzymes in respiration and in energy metabolism. Consequently, the protein content of the mitochondrial fraction is much higher after DEHP administration in comparison with the control but the specific activity of cytochrome *c* oxidase is not elevated (Table 2). An increase of cytochrome *c* oxidase activity is apparent after MEHP and PA administration. The main event occurring in mitochondria when rats were kept on a DEHP-containing diet is the 30-fold increase of carnitine-acetyl transferase determined by a spectrophotometric method. MEHP increases this activity 10 times while the other metabolites have no effect.

The endoplasmic reticulum displays a hypertrophy under the influence of many drugs and chemical reagents and it was interesting to determine if phthalate metabolites affect these membranes. Both enzymes of the NADPH electron transport system, NADPH-cytochrome *c* reductase activity and the amount of cytochrome P-450, increase about 50% with DEHP and MEHP (Table 3). The other three metabolites tested in these experiments did not change the amount or composition of the microsomal membranes. The experiments demonstrate that hydroxylation of

phthalate esters performed by microsomal enzymes may have a role in the production of active metabolites influencing biosynthetic processes.

Parallel to the above chemical analyses, all livers were investigated electron microscopically. The results were clear from the point of view of morphological appearance. The pictures were dominated by proliferating peroxisomes and mitochondria in the case of DEHP and MEHP, without induction of the endoplasmic reticulum. On the other hand, no induction of the intracellular membranes could be observed with EHB, PA or EH, and the pictures were in all respects very similar to that of the control.

The experiments described demonstrate that among the metabolites of DEHP studied here only MEHP has a clear effect and induces both peroxisomes and mitochondria. The 2-ethylhexanol side chain and the phthalic acid are ineffective as inducers of membranes and enzymes. Interestingly, the presence of a non-substituted carboxyl group of the monophthalate ester is of basic importance in obtaining the selective and combined effects on peroxisomes and mitochondria. The induction of hydroxylating enzymes in the case of DEHP and MEHP suggests that oxidative metabolism is

probably an important requirement for production of active metabolites and these metabolites should be identified and isolated in the future.

Acknowledgements. This work was supported by The Swedish Products Control Board, The Swedish Council for Planning and Coordination of Research and The Swedish Medical Research Council.

1. Autian, J. *Environ. Health Persp.* 4 (1973) 3.
2. Thomas, J., Darby, T., Wallin, R., Garvin, P. and Martis, F. *Toxicol. Appl. Pharmacol.* 45 (1978) 1.
3. Ganning, A. E. and Dallner, G. *FEBS Lett.* 130 (1981) 77.
4. Osumi, T. and Hashimoto, T. *J. Biochem.* 85 (1979) 131.
5. Lazarow, P. B. and De Duve, C. *Proc. Natl. Acad. Sci. U.S.A.* 73 (1976) 2043.

Received February 2, 1982.