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IDENTIFICATION OF THE METABOLITES OF SIMPLE PHTHALATE DIESTERS IN RAT URINE

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SUMMARY

Rat urinary metabolites of orally-administered dimethyl, di-*n*-butyl and di-*n*-octyl phthalates have been isolated by high-pressure liquid chromatography on Porasil A. A linear gradient of tetrahydrofuran into *n*-heptane resolved the following classes of diazomethane-treated metabolites: diesters, triesters, ketodiesters, and two classes of hydroxydiesters.

Phthalic acid was a very minor metabolite except in the case of dimethyl phthalate precursor. The corresponding monoesters become more significant as they become more polar (methyl > butyl > *n*-octyl ≈ ethylhexyl). The remaining metabolites are those that would be expected from $\omega-1$ and ω- followed by α- and β-oxidation of the monoesters. Intact diester was excreted as a trace component when dibutyl phthalate was fed, and as a significant component when dimethyl phthalate was fed.

INTRODUCTION

The world production of plasticizer phthalate esters now exceeds three-quarters of a million tons per year[1]. Speakers at a conference on phthalate esters held in 1972 emphasized that relatively little is known about the metabolic fate of these materials, and that they do find their way into the human body[2]. The phthalate moiety of the most commonly used plasticizer, di(2-ethylhexyl) phthalate (DEHP), is known to be rapidly excreted by rats in urine and feces[3].

We have previously reported on the isolation and characterization of the DEHP metabolites excreted in rat urine following oral administration[4]. That study is extended in the present paper to rat urinary metabolites of dimethyl, di-*n*-butyl, and di-*n*-octyl phthalates.

MATERIALS AND METHODS

The basic protocol was as described previously[4]. 0.2-ml doses of dibutyl or dioctyl phthalate, or 0.1-ml doses of the more toxic[1] dimethyl phthalate were adminis-

tered by gavage at 24-h intervals to adult male CD rats (Charles River). Urine was collected over thymol for 48 h following the initial dose of phthalate ester.

Sources of reference compounds and synthetic methods for the reference phthalate monoesters, the method of extracting the metabolites, criteria for deciding that conjugates were absent, methods for obtaining and criteria for interpreting IR, UV, proton magnetic resonance (PMR), and both electron impact (EI) (70 eV) and chemical ionization (CI) (methane) mass spectra have been described in detail previously[4].

Formation of methyl esters with diazomethane and conversion of hydroxyl groups to trimethylsilyl (TMS) ethers was as described previously[4], as was determination of retention indices by gas–liquid chromatography (GLC) on OV-3 silicone. Additionally, hydroxyl groups were allowed to react with dimethylformamide (DMF)–hexamethyldisilazane (HMDS)–trimethyl chlorosilane (TMCS) (4:2:1) for 15 min at 22°; this reagent does not enolize ketone groups, and reacts noticeably more slowly with secondary than with primary alcohols. Ketones were reduced to secondary alcohols by a solution of $NaBH_4$ (2 mg/ml) in 95% ethanol, 15 min at 22°.

Individual metabolites or, in one case, individual homologous series of metabolites were preparatively isolated by pressure-assisted liquid chromatography after treating the urine extracts with diazomethane. A Waters Associates Model ALC 202 chromatograph equipped with a 254-nm differential UV detector, a Model 660 solvent programmer and a 10-mV recorder was used. Samples of the diazomethane-treated urine extracts were injected onto a 61 cm × 2 mm I.D. column of Porasil A-60. Solvent, flowing at 3 ml/min, was linearly programmed in composition from 100% *n*-heptane to 50% heptane–50% tetrahydrofuran in 30 min, with both pumps maintaining 1000 p.s.i. of pressure. Fractions were collected manually as directed by the UV monitor.

Urine extracts before diazomethane esterification were examined by thin-layer chromatography (TLC) on 250-μ layers of silica gel GF (Brinkman, Westbury, N.Y., U.S.A.), developed in unlined tanks with chloroform–methanol–acetic acid (143:7:1.5). Spots were visualized by their quenching of the fluorescent background, and by spraying with acidic dinitrophenylhydrazine[5].

Extracts from urine of rats fed dimethyl phthalate were treated with "Butyl-8" (Pierce, Rockford, Ill., U.S.A.) in order to have gas-chromatographable derivatives that would not be confused with the precursor fed.

RESULTS AND DISCUSSION

Dimethyl phthalate

The 24-h urine contained an average of 44.6 mole per cent of the phthalate moiety fed, based on a molar extinction coefficient of 1.14×10^3 in 1,2-dimethoxyethane at 278 nm. The only detectable metabolites were free phthalic acid (14.4%) and monomethyl phthalate (77.5%). In addition, a small amount of dimethyl phthalate (8.1%) was excreted intact. The above are mole percentages of recovered phthalate.

These products were identified by co-chromatography with appropriate standards after conversion of the free carboxyl groups to butyl esters with Butyl-8. The derivatized excretion products co-chromatographed with the above named standards during TLC in petroleum ether (b.p. 30–60°)–diethyl ether–acetic acid (80:20:1) and

during GLC on OV-3 (ref. 4). In addition, their mass spectra matched those of the reference compounds.

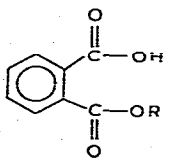
Di-n-butyl phthalate (DBP)

The only product having UV absorption above 230 nm after KOH hydrolysis of the extracted metabolites was found to be unsubstituted phthalic acid, as was the case for metabolites of diethylhexyl phthalate[4] and of di-*n*-octyl phthalate (following). Accordingly, urine components were identified as DBP metabolites if they met the following criteria: (a) they were not present in urine of rats not fed DBP, (b) they absorbed maximally at 278 nm in dimethoxyethane, and (c) mass spectra of the diazomethane-treated materials showed major peaks at 149 a.m.u. and, for phthalates having at least one methyl ester group, 163 a.m.u. In the mass spectra of all phthalates[6] *m/e* 149 appears and is usually the base peak. Methyl phthalates, however, usually have the base peak at 163 a.m.u.[4]. There were no compounds detected having a 149 a.m.u. mass spectral peak that did not absorb maximally at 278 nm in this study.

A total of six metabolites of DBP were detected in the urine, along with a trace (0.1 %) of intact DBP. All six could be resolved by high-pressure liquid chromatography, with each fraction giving a distinct peak on OV-3 (GLC). Chromatographic and some of the spectral properties of the metabolites are summarized in Table I. Only a total of 24.6 % of the phthalate moiety fed was recovered in the urine by 24 h after the second and by 48 h after the first feeding.

Metabolite DBP-3 was identified as free phthalic acid by comparison of its chromatographic mobilities before and after esterification, the IR spectrum of its methyl ester and the EI and CI mass spectra of its methyl ester with those of similarly treated known phthalic acid. Dibutyl phthalate and monobutyl phthalate (DBP-1 and DBP-2, respectively) were also identified by direct comparison with reference standards.

Metabolites DBP-4, -5, -6, and -7 were not extracted from urine at pH 8 but were extracted at pH 2. This and the observation that their TLC mobilities increased markedly upon reaction with diazomethane indicated part structures of:



where R remained to be determined. DBP-5 reacted with acidic dinitrophenylhydrazine on a TLC plate, suggesting an aldehyde or ketone. Its methyl ester formed a TMS derivative when it was heated with bis(trimethylsilyl)trifluoroacetamide and trimethylchlorosilane[4], but not when DMF–HMDS–TMCS was used: this suggested the formation of an enol ether. The IR spectrum of methyl DBP-5 did not show aldehyde, –C(O)–H, at 3.6 μ, but the carbonyl peak at $\sim$ 1720 cm^{-1} was usually broad for a phthalate ester. The molecular weight of the methyl ester was 250, and this changed to 252 on treatment with borohydride. Finally, the CI mass spectrum showed a base peak at 208 a.m.u. (MH-43) corresponding to cleavage of $-C(O)-CH_3$ from the side chain, a possible event for methyl ketones. In the CI mass spectrum *m/e* 71, 15% abundance, was also found as would be expected for $R=[-CH_2CH_2COCH_3]^+$.

TABLE I

PROPERTIES OF DI-*n*-BUTYL PHTHALATE METABOLITES

Metabolite No.	*Percentage of phthalate excreted*	*R.I. of methyl ester*[a]	*R.I. of TMS ether*[b]	*TLC R_F of free acid*[c]	*TLC R_F of methyl ester*[d]	*Porasil elution volume (ml)*	*IR C=O ($\lambda_{max.}$)*[e]	*IR C-OH ($\lambda_{max.}$)*[e]	*Mol. wt.*[f]	*m/e of R*[f]	*m/e of TMS fragment*[g]	*Other useful fragment*[f]
1	0.4	1993		0.85	0.80	4.5	1720		278			M − 73 (63%)
2	89.8	1773		0.45	0.67	15.0	1720		236	–	–	M − 73 (100%)
3	2.7	1523	–	0.02	0.47	18.0	1720		194	–	–	M − 31 (100%)
4	3.5	2080		0.22	0.19	27.0	1720, 1735		280	101 (99%)		
5[h]	0.5	1941	1990[i]	0.40	0.23	35.5	1718, broad		250[j]	71 (15%)		M − 42 (100%)
6	3.0	1955	2013	0.36	0.22	48.0	1720	1105	252	73 (4%)	117 (35%)	M − 17 (13%)
7	0.1	2059	2109	0.32	0.13	57.0	1720	1050	252	73 (1%)	89 (43%) 91 (17%)	M − 17 (7%)

[a] Retention index of diazomethane-treated metabolite on OV-3 at 180°.
[b] Retention index of methyl ester TMS ether derivative on OV-3 at 180°.
[c] TLC of total urine extract (from urine at pH 2) on silica gel GF (250 μ) in chloroform–methanol–acetic acid (143:7:1.5).
[d] TLC of diazomethane-treated extract on silica gel GF (250 μ) in light petroleum ether (b.p. 30–60°)–diethyl ether–acetic acid (120:30:2).
[e] IR spectra run as neat films on NaCl with a combination prism/grating instrument. Values in cm^{-1} given for diazomethane-treated material.
[f] From methane CI mass spectra, which showed M + 1, M + 29 and M + 41 recombination fragments, major peaks for R (see text), and relatively few elimination reactions, 149 and 163 a.m.u. were either base peaks or very major peaks in all cases. Samples run as methyl esters.
[g] From 70-eV EI mass spectra of the trimethylsilylated methyl esters. 91 a.m.u. = $(CH_3)_3SiOH_2^+$; 117 a.m.u. = $(CH_3)_3SiOC^+HCH_3$.
[h] Forms visible derivative with acidic dinitrophenylhydrazine spray on TLC plate[5].
[i] Enol ether.
[j] Changes to 252 after treatment of the methyl ester with $NaBH_4$.

Borohydride reduction of methyl DBP-5 gave a compound indistinguishable from methyl DBP-6. The latter had a free hydroxyl group as evidenced by its facile and quantitative formation of a TMS derivative. The C–OH band was at 1105 cm^{-1} in its IR spectrum, suggesting a secondary alcohol. M−17, 13% (M + 1 − 18, 235 a.m.u.) and *m/e* = 73, 4%, in the CI mass spectrum of methyl DBP-6 confirmed an alcohol structure, while the high abundance of *m/e* = 117 (cleavage of trimethylsilylethanol) in the EI mass spectrum of the TMS derivative confirmed a secondary alcohol.

Metabolites DBP-5 and DBP-6 then could be identified as having R = $-(CH_2)_2COCH_3$ and $-(CH_2)_2CHOHCH_3$, respectively.

Metabolite DBP-7 was more polar than DBP-6, but had the same molecular weight and a very similar CI mass spectrum. It also readily formed a TMS derivative. However, the IR spectrum showed C–OH at 1050 cm^{-1} and the EI mass spectrum of the TMS derivative showed a negligible peak at 117 a.m.u. Instead, *m/e* 89 (43%) and 91 (17%) were prominent. These findings were consistent with identification of the R group of DBP-7 as $-(CH_2)_3CH_2OH$.

The diazomethane-treated metabolite DBP-4 showed split carbonyl absorption in the IR spectrum at 1720 and 1735 cm^{-1}, indicating both aromatic and aliphatic ester groups. Its molecular weight of 280 and prominent 101 a.m.u. peak in the CI mass spectrum, along with non-reactivity toward silylation reagents identified the R group of DBP-4 as $-(CH_2)_3COOH$, and $-(CH_2)_3COOCH_3$ after treatment with diazomethane.

Di-n-octyl phthalate (DOP)

In previous work[4] it was possible to separate the metabolites of di(2-ethylhexyl) phthalate by preparative TLC. That approach failed with the di-*n*-octyl phthalate metabolites because of the presence of a seven-membered homologous series among them, which produced a very elongated TLC migration zone. This series of compounds, however, after esterification with diazomethane, eluted together from Porasil A-60 as a well resolved, sharp peak.

The methylated DOP metabolites are summarized in Table II. Dimethyl phthalate (from free phthalic acid) was again detected (DOP-2); it and methyl octyl phthalate (DOP-1) (from monooctyl phthalate) were identified by direct comparison of chromatographic and spectral properties with known standards. Intact dioctyl phthalate was not detected.

DOP-3 through DOP-9 are eluted together from Porasil. This diazomethane-treated fraction had carbonyl absorption at both 1720 and 1735 cm^{-1}, and the major component of the fraction was identical in mass spectrum and retention index to the previously identified methyl DBP-4. Thus this series of compounds had R = $-(CH_2)_nCOOH$, where n = 1 to 7. Perhaps significantly, the compounds for which n = 1 and n = 2 were present in almost negligible amounts. This mixture of compounds could be partially purified by TLC of the acids before treatment with diazomethane, although it was contaminated with DOP-11 and DOP-12. Proton magnetic resonance spectra revealed that the TLC-purified mixture acquired aromatic and aliphatic methyl ester moieties (singlets at δ = 3.96 and 3.7 ppm, respectively) only after treatment with diazomethane. In fact, this observation applied to the total urine extract, which contained 31.0% of the phthalate moiety fed.

TABLE II

PROPERTIES OF DI-*n*-OCTYL PHTHALATE METABOLITES

Parameter	*DOP metabolite No.*											
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*
Area, %	0,1	2,6	0,1	0,1	61,7	0,2	0,6	1,7	8,0	11,5	10,8	2,7
R.I. of methyl ester[a]	2193	1523	1870	1976	2080	2180	2282	2383	2483	2365	2376	2442
R.I. of TMS derivative[b]	...	...	...	...	...	...	...	...	...	2408[b]	2428	2502
TLC R_f, of free acid[c]	0.59	0,02	0,22[i]	0,22[i]	0,22[i]	0,22[i]	0,22[i]	0,22[i]	0,22[i]	0,51	0,30	0,25
TLC R_f, of methyl ester[d]	0,69	0,48	0,27[i]	0,27[i]	0,27[i]	0,27[i]	0,27[i]	0,27[i]	0,27[i]	0,21	0,12	0,06
IR C=O, $\lambda_{max.}$[e]	1720	1720	1720, 1735[j]	1720, 1735[j]	1720, 1735[j]	1720, 1735[j]	1720, 1735[j]	1720, 1735[j]	1720, 1735[j]	1718 broad	1720	1720
IR C-OH, $\lambda_{max.}$[e]	...	...	...	...	...	...	...	...	...	...	1105	1051
Mol. wt.[f]	292	194	252	266	280	294	308	322	336	306	308	308
R, a.m.u. (%)[f]	113 (14)			87 (15)	101 (98)	115 (100)	129 (87)	143 (68)	157 (79)	127 (82)	129 (9)	129 (15)
TMS fragment, a.m.u. (%)[g]										...	117 (40)	91 (100)
Other useful fragment (%)[f]		M−31 (100)	M−31 (55)	M−31 (14)			M−31 (1)	M−31 (11)	M−31 (15)	M−57 (6)	M−17 (27)	M−17 (20)

[a]–[g] See footnotes to Table I.

[i] Enol ether.

[j] Center of a streak.

[j] Run as mixture (homologous series).

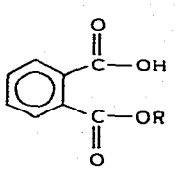
On GLC and mass spectral evidence, DOP-10 methyl ester was converted to DOP-11 methyl ester on treatment with borohydride. Once again the spectral properties of methyl DOP-10 were consistent with a methyl ketone structure, as were observations that DOP-10 reacted with acidic dinitrophenylhydrazine and gave a positive micro-iodoform reaction[7]. The NMR spectrum of methyl DOP-10 showed four aromatic protons (δ = 7–8), three aromatic methyl ester protons (singlet at δ = 3.96), two protons assigned to Ar-C(O)-O-CH_2- (triplet at 4.38 ppm, J = 5 Hz), three protons assigned to -C(O)-CH_3 (singlet at 2.18 ppm), and a total of ten protons in various other -CH_2- environments. The R group of DOP-10 was thus conclusively identified as -$(CH_2)_6COCH_3$, implying that DOP-11 had R = -$(CH_2)_6CHOHCH_3$. The latter was confirmed by the mass spectral data in Table II, especially the intense peak at *m/e* 117 in the 70-eV mass spectrum of the TMS ether. Again, the replacement of the *m/e* 117 peak by one at *m/e* 91 in the 70-eV mass spectrum of the TMS ether of DOP-12 indicated that this metabolite had R = -$(CH_2)_7CH_2OH$, confirmed by its IR spectrum (C-OH at 1051 cm^{-1}).

All of the discussed metabolites of both dibutyl and dioctyl phthalate were excreted in the form of phthalate half esters as evidenced by the appearance of Ar-C(O)-OCH_3 peaks in the PMR spectra only after diazomethanolysis. Similarly, aliphatic methyl ester peaks were not seen in the PMR spectra until after diazomethane treatment. Thus the R groups of the various excreted metabolites could be listed as

TABLE III

IDENTITIES OF THE METABOLITES AS EXCRETED

Metabolite	*R**
DBP-1	-$(CH_2)_3CH_3$ = 2 (intact DBP)
DBP-2	-$(CH_2)_3CH_3$ (monobutyl phthalate)
DBP-3	-H (phthalic acid)
DBP-4	-$(CH_2)_3COOH$
DBP-5	-$(CH_2)_2COCH_3$
DBP-6	-$(CH_2)_2CHOHCH_3$
DBP-7	-$(CH_2)_3CH_2OH$
DOP-1	-$(CH_2)_7CH_3$ (monooctyl phthalate)
DOP-2	-H (phthalic acid)
DOP-3	-CH_2COOH
DOP-4	-$(CH_2)_2COOH$
DOP-5	-$(CH_2)_3COOH$ (= DBP-4)
DOP-6	-$(CH_2)_4COOH$
DOP-7	-$(CH_2)_5COOH$
DOP-8	-$(CH_2)_6COOH$
DOP-9	-$(CH_2)_7COOH$
DOP-10	-$(CH_2)_6COCH_3$
DOP-11	-$(CH_2)_6CHOHCH_3$
DOP-12	-$(CH_2)_7CH_2OH$

* From 

in Table III. CI mass spectra of all the DBP and DOP metabolites are given in Tables IV and V.

The excreted metabolites from dimethyl phthalate precursor could readily be quantitated, since appropriate pure standards were available. Quantifying the DBP and DOP metabolites, however, was somewhat more arbitrary. Although the UV monitor output from the high-pressure liquid chromatograph has been used for quantitation[5], we questioned whether this approach would be reliable for a total urine extract when the monitor could not be set at λ_{max} for phthalates. The GC–mass spectrometer's total ion monitor might be expected to have differing responses to different compounds, as would also be true for the hydrogen flame ionization detector. However, we found that the three approaches to quantitation gave quite similar results, and therefore the percentages given in Tables I and II are the averages of peak area percentages from the flame detector, ion monitor and UV monitor applied to the diazomethane-treated total urine extracts, setting the combined area of peaks assigned to phthalates equal to 100% in all cases.

TABLE IV

METHANE CI MASS SPECTRA OF DBP METABOLITES*

Fragment	*Per cent abundance in spectra of methyl ester of*						
	DBP-1	*DBP-2*	*DBP-3*	*DBP-4*	*DBP-5*	*DBP-6*	*DBP-7*
M + 41**	2	6.5	3	0.5	0.5	0.5	1
M + 29**	6	13	9	8	1	3	4
M + 1**	12.5	39	18	2	8.5	7.5	10
M − 17	—	—	—	—	—	12.5	7
M − 31	5	29	100	—	4	6	5
M − 42	—	—	—	—	100	—	—
M − 71	—	—	—	—	20.5	—	—
M − 73	62	—	—	—	—	2	2
m/e 181	6	4	0.1	1	2	12	8
m/e 163	50	100	100	100	6	100	100
m/e 149	100	25.5	3.5	1	8.5	14	10
m/e 101	—	—	—	99	—	—	—

* 60–500 a.m.u. scans.

** Recombination fragments, M = mol. ion.

Reliable identification of these compounds was greatly facilitated by the simplicity of the methane CI mass spectra, especially the tendency of these spectra to provide a relatively major peak corresponding in *m/e* to the intact R group as discussed above. In addition, many of the minor metabolites would have been overlooked without the capability of the computerized system to search for individual (149 and 163) mass ions.

The only occurring metabolism of dimethyl phthalate appeared to be hydrolysis of one or both ester groups. Dibutyl phthalate was predominantly metabolized by hydrolysis of one ester bond and both terminal (ω) and subterminal ($\omega-1$) oxidation of the remaining alkyl chain. As is the case with ω- and $\omega-1$-oxidation of fatty acids[8], the resulting primary and secondary alcohols were apparently further oxidized to acid and ketone, respectively. In the case of dioctyl phthalate, the longer

TABLE V

METHANE CI MASS SPECTRA OF DOP METABOLITES*

Fragment	*Per cent relative abundance in spectra of methyl ester of*											
	DOP-1	*DOP-2*	*DOP-3*	*DOP-4*	*DOP-5*	*DOP-6*	*DOP-7*	*DOP-8*	*DOP-9*	*DOP-10*	*DOP-11*	*DOP-12*
M + 41[b]	2	3	4	3	0,5	1	1	3	3	3	1	1
M + 29[b]	6	9	8	9	7,5	8	8	11,5	11,5	12	4	5
M + 1[b]	68	18	7	4	2,5	2,8	6	15	12	12,5	10,7	10,5
M - 17	-	-	-	-	-	-	-	-	-	-	27	20
M - 31	1	100	55	4	-	-	1	11	15	14	8	10
M - 57	-	-	-	-	-	-	-	-	-	6	-	-
m/e 181	41	0,1	1	0,5	0,7	1	3,5	3,5	7,5	2	15	16
m/e 163	100	100	100	100	100	85	100	100	100	100	100	100
m/e 157	-	-	-	-	-	-	-	-	79	-	-	-
m/e 149	18	3,5	1	0,5	1	2	1	2,5	4,5	2	8,5	9
m/e 143	-	-	-	-	-	-	-	67,5	-	-	-	-
m/e 129	-	-	-	-	-	-	86,5	-	-	-	9,3	15
m/e 127	-	-	-	1	-	1	3	8	-	82	-	-
m/e 115	-	-	-	-	-	100	-	-	-	-	-	-
m/e 111	-	-	-	-	-	-	-	4	-	-	21	17
m/e 101	-	-	-	-	98	9,5	-	-	-	-	-	-
m/e 87	-	-	-	15	1,5	17	-	-	-	-	-	-

* 60–500 a.m.u. scans.

** Recombination fragments from M + H^+, M + $C_2H_5^+$, M + $C_3H_5^+$.

alkyl side chain permitted a series of α- and β-oxidations of the carboxyl-terminated metabolites. We have previously postulated a similar sequence of events in the metabolism of diethylhexyl phthalate by rats[4]. It remains to be seen whether or not the same enzymes are involved in ω- and $\omega-1$-oxidation of phthalate monoesters as are involved in ω- and $\omega-1$-oxidation of fatty acids.

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