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BIOCHEMICAL STUDIES ON PHTHALIC ESTERS—II.

EFFECTS OF PHTHALIC ESTERS ON MITOCHONDRIAL RESPIRATION OF RAT LIVER

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Abstract—The effects of dialkyl and monoalkyl phthalates and some related compounds on mitochondrial respiration of rat liver were investigated. In a series of dialkyl phthalates, di-*n*-propyl phthalate showed the most remarkable inhibition on the state-3 respiration. The inhibitory effects were reduced with either decreasing or increasing chain length for this compound. Di-(2-ethylhexyl)phthalate had no effect. On the other hand, in a series of monoalkyl phthalates the inhibition was increased as the alkyl group was lengthened up to heptyl. Relationship between chemical structure and inhibitory effect was also studied using some phthalate analogs and related compounds. It was shown that the length of alkyl chains was more important than aromatic ring structure for the inhibitory effects of phthalates. Mechanisms of inhibitory effects by phthalates were discussed, and it was suggested that dialkyl and monoalkyl phthalates mainly acted as energy-transfer inhibitors and blocked the point before the DNP-sensitive site in the energy-transfer pathway.

Because of the wide distribution of phthalic ester plasticizers in our environment, there are many possible pathways through which these compounds can be introduced into the human body. So, recently considerable attention has been paid to the biological effects of these chemicals, especially of di-(2-ethylhexyl)phthalate (DEHP), a representative plasticizer.

On the distribution studies of DEHP in rats, some investigators reported preferential localization of phthalate in the liver in a short time after intravenous injection [1–6]. The same localization was also observed in feeding studies [6, 7]. In other experiments with rats, it was observed that high dosages of DEHP were associated with growth retardation and increase of liver weight [6, 8–12].

Most of the urinary metabolites of DEHP were those to be expected from the ω- or ω-1 oxidation and the subsequent β-oxidation of the alkyl side chain of mono-(2-ethylhexyl)phthalate (MEHP) [6, 13]. These metabolic changes are believed to occur mainly in the liver.

On the other hand, Nazir *et al.* have observed the specific localization of DEHP in bovine heart muscle mitochondria [14], although the biological significance of this phenomenon is not clear. Recently, Ohyama reported the effect of dialkyl phthalates on the respiration of rat liver mitochondria and concluded that phthalic esters were electron and energy transport inhibitors but not uncouplers [15].

This paper deals with the effects of dialkyl and monoalkyl phthalates on mitochondrial respiration of rat liver.

Abbreviations: DEHP, di-(2-ethylhexyl)phthalate; MEHP, mono-(2-ethylhexyl)phthalate; BPBG, butylphthalyl butylglycolate; DBP, di-*n*-butylphthalate; DPP, di-*n*-propylphthalate; DNP, 2,4-dinitrophenol.

MATERIALS AND METHODS

Preparation of mitochondrial fraction. Mitochondrial fraction was prepared from the liver of male Wistar rats (150–250 g), according to the method modified by Hagihara [16]. The medium for this preparation contained 0.21 M mannitol, 75 mM sucrose, 10 mM Tris–HCl buffer (pH 7.4) and 0.1 mM EDTA. One ml of the final suspension contained the mitochondrial fraction isolated from 1 g of wet liver.

Protein determination. Protein was determined by the Biuret reaction [17] using crystalline bovine serum albumin as a standard.

Assay of oxidative phosphorylation. In some cases, the oxygen consumption was measured by the Warburg manometric technique in air as the gas phase. The main compartment contained 75 mM Tris–HCl buffer (pH 7.4), 20 mM phosphate buffer (pH 7.4), 10 mM KCl, 5 mM $MgCl_2$, 10 mM KF, 2.5 mM ATP, 1 mM phthalates (in ethanol) and 0.2 ml of mitochondrial suspension. The final concentration of ethanol was 2%. The reaction volume was adjusted to 2.0 ml with 0.25 M sucrose. The center well contained 0.2 ml of 20% (w/v) KOH. After equilibration for 10 min at 30°, the reaction was started by tipping in 20 mM sodium succinate, 20 mM glucose and 1 mg of hexokinase from a side-arm. After measurement of the O_2 uptake for 10 min, the reaction was stopped by the addition of trichloroacetic acid in final concentration of 5% (w/v). Orthophosphate remaining in the supernatant was determined by the method of Fiske and Subbarow [18]. The disappearance of phosphate was taken as a measure of the amount of high-energy bond formation. However, in most cases, the oxygen consumption was measured polarographically by an oxygen electrode apparatus with a rotating platinum electrode (Model PO 100A, Yanagimoto Co. Ltd.) at 25° The reaction vessel contained 0.25 M mannitol,

Table 1. Effects of dialkyl phthalates on mitochondrial respiration by manometric determination

Phthalate	Number of experiments	Uptake of O_2 (μatoms/10 min)	Uptake of P (μmoles/10 min)	P:O
	25	6.54 ± 0.14*	11.26 ± 0.20	1.72 ± 0.03
Dimethyl-	8	5.84 ± 0.33	9.10 ± 0.42	1.56 ± 0.05
Diethyl-	8	4.03 ± 0.17	5.10 ± 0.48	1.27 ± 0.10
Di-*n*-propyl-	8	3.36 ± 0.20	0.69 ± 0.38	1.21 ± 0.10
Di-*n*-butyl-	8	5.33 ± 0.37	2.21 ± 0.54	0.41 ± 0.09
Di-*n*-amyl-	8	5.49 ± 0.29	6.89 ± 0.46	1.27 ± 0.08
Di-*n*-hexyl-	10	5.39 ± 0.22	8.33 ± 0.23	1.56 ± 0.04
Di-*n*-heptyl-	10	5.90 ± 0.24	9.48 ± 0.52	1.60 ± 0.04
Di-*n*-octyl-	10	6.13 ± 0.24	9.68 ± 0.37	1.58 ± 0.05
Di-(2-ethylhexyl)-	10	6.56 ± 0.25	10.97 ± 0.57	1.67 ± 0.06
BPBG	8	5.35 ± 0.34	7.14 ± 0.55	1.33 ± 0.07

* Values represent means ± S. E. for the number of experiments listed.

10 mM Tris–HCl buffer (pH 7.4), 10 mM phosphate buffer (pH 7.4), 10 mM KCl, 2 mM $MgCl_2$, 0.2 mM EDTA and 2.0–2.8 mg of mitochondrial protein. The total volume of reaction mixture was 2 ml. The state-4 and state-3 respirations were initiated by the addition of 3 mM sodium succinate and 375 μM ADP respectively. One mM phthalates (in ethanol) was added at each state. The final concentration of ethanol was 1%. The respiratory control ratio (R.C.) was obtained as the ratio of the respiration rate of state-3 to that of state-4, and the phosphorylative activity (ADP/O ratio) was calculated from the concentration of ADP and oxygen consumed at state-3 as described by Chance *et al.* [19].

Reagents. MEHP and phthalic monoesters from methyl to *n*-octyl, except for ethyl ester, were synthesized by condensation of the corresponding alcohols with phthalic anhydride [20], and its chemical purity was checked by gas chromatography after methylation. Dialkyl phthalates and monoethyl phthalate were purchased from Tokyo Kasei Co., butyl phthalyl butyl glycolate (BPBG) and hexokinase from Wako chemical Co., ATP from Sigma, ADP from P-L Biochemicals, and oligomycin was the kind gift of the late Dr. S. Muraoka. Other chemicals were of the highest purity commercially available.

RESULTS

Effects of dialkyl phthalates on mitochondrial respiration. In Table 1 are shown the effects of dialkyl phthalates on mitochondrial respiration and oxidative phosphorylation measured by the manometric determination. A series of phthalates having alkyl group from methyl to *n*-octyl were tested, and all of them inhibited the O_2 uptake, the phosphorylation and P:O ratio in greater or lesser degree. The inhibition of the phosphorylation was more extensive than that of the O_2 uptake. As the most remarkable example, in the case of di-*n*-propyl phthalate (DPP), the O_2 uptake was inhibited about 50 per cent, and the phosphorylation almost completely. The inhibitions by dialkyl phthalates were enhanced sharply as the alkyl chains were increased from methyl to propyl, but reduced reversely as the chains further increased from butyl to octyl. It is noteworthy that the most common plasticizer, DEHP, showed no inhibition. The inhibition by BPBG was the same extent as di-*n*-amyl phthalate.

As shown in Table 2, the inhibitory effects of dialkyl phthalates on the state-3 respiration measured by the polarographic method were similar to the above-mentioned manometric results in the order of the length of alkyl chains, but the degree of the inhibition was generally more potent. On the other hand, the state-4 respiration was scarcely inhibited, or rather stimulated in some cases. Di-*n*-butyl phthalate (DBP) stimulated considerably the state-4 respiration and inhibited the state-3 respiration, but DPP showed little effect on the state-4 respiration and strongly inhibited the state-3 respiration. In both DBP and DPP the respiratory controls were lost completely. The inhibi-

Table 2. Effects of dialkyl phthalates on mitochondrial respiration by polarographic determination

Phthalate	Uptake of O_2 (μM/min) State-4	State-3	R.C.	ADP:O
	16	77	4.81	1.69
Dimethyl-	16	60	3.75	1.68
Diethyl-	15	32	2.13	1.66
Di-*n*-propyl-	17	17	1.0	
Di-*n*-butyl-	30	30	1.0	
Di-*n*-amyl-	15	42	2.80	1.54
Di-*n*-hexyl-	16	66	4.13	1.69
Di-*n*-heptyl-	16	69	4.31	1.69
Di-*n*-octyl-	17	70	4.11	1.68
Di-(2-ethylhexyl)-	16	75	4.69	1.72
BPBG	17	46	2.71	1.55

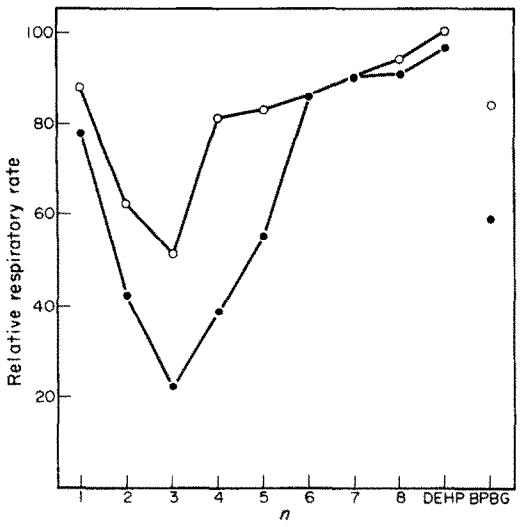


Fig. 1. Inhibition of mitochondrial respiration by dialkyl phthalates. Relative respiratory rate means the ratio of the respiratory rate in the presence of phthalates to that in the absence of phthalates. O——O, manometric determination; ●——●, polarographic determination (state-3); n, carbon number of alkyl chain in dialkyl phthalate.

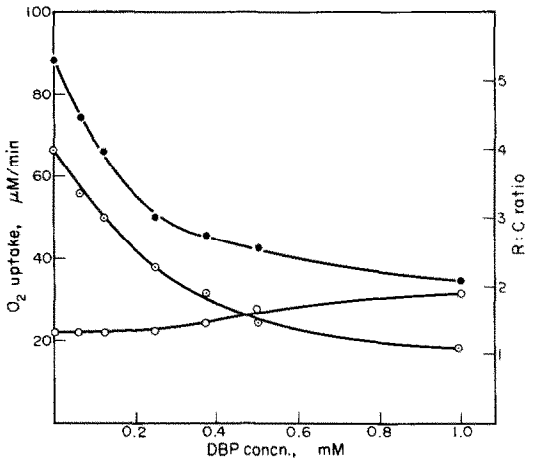


Fig. 2. Effect of DBP concentration on respiration and respiratory control ratio. ●——●, state-3 oxygen uptake; O——O, state-4 oxygen uptake; ⊙- - -⊙, respiratory control ratio.

tory effects of dialkyl phthalates measured by two methods are summarized in Fig. 1.

Figure 2 shows the effect of DBP concentration on the respiration. The state-3 respiration and the respiratory control ratio were decreased with increasing the concentration of DBP, but the stimulation of the state-4 respiration was not so severely affected as in the case of the state-3 respiration.

In Fig. 3, the inhibition of respiration by DBP was compared with the effects of 2,4-dinitrophenol (DNP) and oligomycin. Oligomycin inhibited the state-3 respiration to the level of state-4, and the inhibition was reversed by DNP. The inhibition of the state-3 respiration by DBP exhibited little difference from that by oligomycin in the time course of the inhibition and it was not reversed by DNP (Fig. 3a). This result suggests that DBP has the property somewhat different from a typical energy transfer inhibitor and its blocking site is located before the DNP-sensitive site. Oligomycin had no effect on DNP-stimulated respiration, but DBP showed the inhibition which was comparable to its inhibitory effect on the state-3 respiration (Fig. 3b). This observation also supports the assumption concerning the blocking site of DBP described above.

Effects of some related compounds. In order to elucidate the correlation between the inhibitory effect and

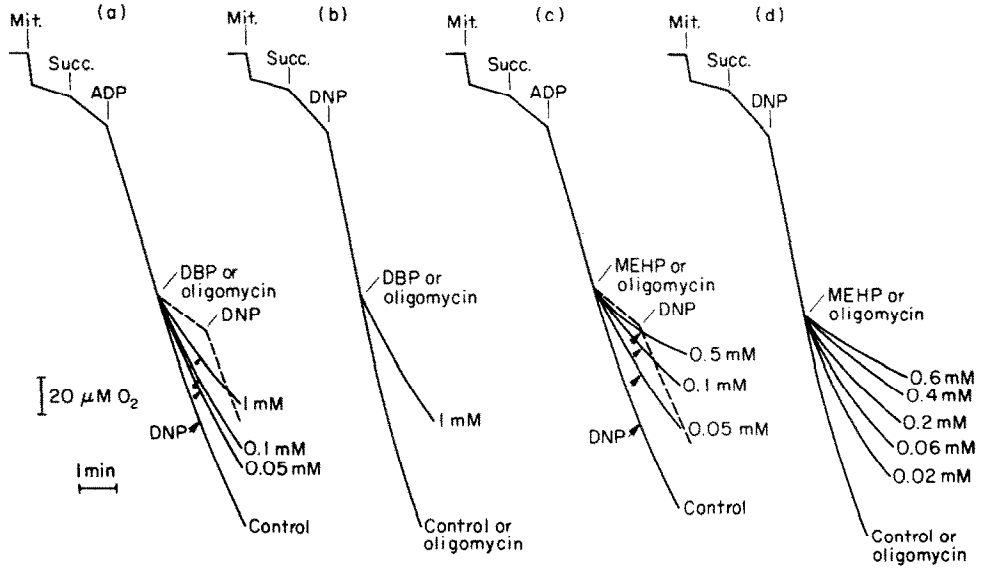
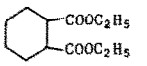
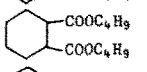
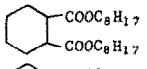
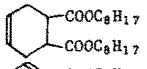
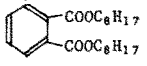
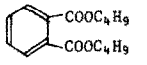
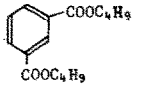
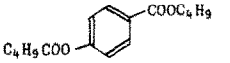
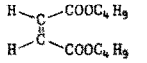
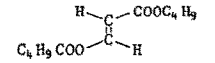
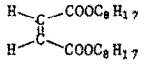
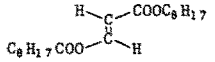
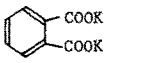


Fig. 3. Polarographic traces showing oxygen utilization by liver mitochondria. Each composite curve consists of a series of superimposed traces of individual experiments in which the amount of inhibitor added at the points indicated was varied. Traces a and c are comparison of inhibitory effect of DBP or MEHP with oligomycin. Trace showing effect of oligomycin was indicated by the dotted line. At the indicated point, DNP was added to test its effect in releasing the respiratory inhibition. Traces b and d show the effect of DBP or MEHP on the DNP-stimulated respiration. Concentration: DNP, 0.1 mM; oligomycin, 10 μg/ml.

Table 3. Comparison of inhibitory effects of di-(2-ethylhexyl) and di-*n*-butyl esters on mitochondrial respiration

Compound		Inhibition at state-3 (%)	R.C.
Diethyl cyclohexane-1,2-dicarboxylate		16	3.61
Di-*n*-butyl cyclohexane-1,2-dicarboxylate		77	1.29
Di-(2-ethylhexyl) cyclohexane-1,2-dicarboxylate		13	4.18
Di-(2-ethylhexyl) tetrahydro-phthalate		10	4.16
Di-(2-ethylhexyl) phthalate		3	4.69
Di-*n*-butyl phthalate		61	1.0
Di-*n*-butyl isophthalate		53	2.40
Di-*n*-butyl terephthalate		38	3.0
Di-*n*-butyl maleate		45	3.01
Di-*n*-butyl fumarate		3	3.43
Di-(2-ethylhexyl) maleate		17	4.37
Di-(2-ethylhexyl) fumarate		0	4.81
Potassium phthalate		15	3.45
2-ethylhexanol*	$C_4H_9CH(C_2H_5)CH_2OH$	4	4.80

* Concentration was 1%.

chemical structure of phthalic esters, the several related compounds were tested, as seen in Table 3. The inhibitions of the state-3 respiration by dialkyl cyclohexane-1,2-dicarboxylates were similar to those by dialkyl phthalates, in decreasing order for di-*n*-butyl, diethyl and di-(2-ethylhexyl) groups. Di-*n*-butyl cyclohexane-1,2-dicarboxylate was the strongest inhibitor of all the related compounds tested. Di-(2-ethylhexyl) cyclohexane-1,2-dicarboxylate was the most effective, when compared with the other two di-(2-ethylhexyl) esters, that is, of tetrahydrophthalate and of phthalate.

Di-*n*-butyl esters of iso and terephthalic acid were also effective and the inhibitory effects of these three isomers were decreased in the order of DBP, di-*n*-butyl isophthalate and di-*n*-butyl terephthalate.

Di-*n*-butyl maleate, which structure is identical with the partial structure of DBP, inhibited about 50 per cent of the respiration, but the *trans* isomer of this compound, di-*n*-butyl fumarate was little effective. When butyl group of these compounds was replaced by 2-ethylhexyl group, the *cis* isomer was more effective than the *trans* isomer.

Phthalic acid showed some inhibitory effect on respiration, but 2-ethylhexanol gave little effect at the concentration of 1%.

Effects of monoalkyl phthalates. As shown in Table 4, the inhibitory effects of monoalkyl phthalates on the state-3 respiration and the respiratory control ratios were enhanced, and ADP:O ratios were reduced with increasing the length of alkyl chains up to heptyl group. In the case of the monophthalates with alkyl groups longer than butyl, the respiratory control was lost and ADP:O ratio could not be obtained. MEHP showed approximately the same inhibition as mono-*n*-heptyl phthalate, the strongest inhibitor of di- and monoalkyl phthalates tested, and its presence of 100 μM concentration was enough to cause 50 per cent inhibition of the state-3 respiration.

In Figs. 3c and 3d, the inhibition of respiration by MEHP was compared with the effects of DNP and oligomycin as shown in the case of DBP. The inhibitory effect of MEHP was similar to that of DBP but more potent, so that MEHP inhibited the state-3 respiration to the level lower than state-4 at the high

Table 4. Effects of monoalkyl phthalates on mitochondrial respiration

Phthalate	Concn (mM)	Uptake of O_2 (μM/min) State-4	State-3	R.C.	ADP:O
	0	16	63	3.94	1.60
Monomethyl-	1	16	57	3.56	1.60
Monoethyl-	1	17	59	3.47	1.54
Mono-*n*-propyl-	1	17	59	3.47	1.52
Mono-*n*-butyl-	1	19	43	2.26	
Mono-*n*-amyl-	1	16	34	2.13	
Mono-*n*-hexyl-	1	16	24	1.50	
Mono-*n*-heptyl-	1	17	9		
	0.1	18	29	1.61	
Mono-*n*-octyl-	1	16	16	1.0	
	0.1	17	34	2.0	
Mono-(2-ethylhexyl)-	1	15	10		
	0.1	16	28	1.75	

concentration. This result suggests that MEHP shares the property as an inhibitor of electron transport.

DISCUSSION

The toxicological evaluation of dialkyl phthalates has been done by many workers [21–23] and it has been indicated that the inverse relationship existed between the toxicity and the molecular weight, and that the toxicity in experimental animals and tissue culture cells was parallel to their water solubility. However, recently other workers [24, 25] reported the different observation on tissue culture cells from that mentioned above.

The difficulty encountered in our experiment was low solubility of phthalates in aqueous media. Therefore phthalates were dissolved in ethanol and added to the mitochondrial system. However, it has been reported that ethanol was not suitable as a vehicle, since ethanol had some inhibitory effect on the respiration of freshly isolated mitochondria with succinate [26, 27]. In fact this finding agreed with our preliminary experiment. Then, in order to minimize the effect of ethanol, the addition of ethanol was restricted to the final concentration of 1% in most cases, and not exceed 2% in any case.

In our results, the inhibition of mitochondrial respiration by dialkyl phthalates was not always followed in the order of the length of alkyl chains, but the maximum inhibitory effect on the state-3 respiration was observed in DPP.

On the other hand, monoalkyl phthalates exhibited an increase in inhibitory effect with increase in the length of alkyl chains.

It was noteworthy that the inhibitory effect of MEHP, a metabolic intermediate of DEHP, was the strongest among the dialkyl and monoalkyl phthalates tested, whereas DEHP had no effect. This fact reminds us of the report [12] that MEHP produced liver enlargement accompanied by depression of the activity of mitochondrial enzyme such as succinate dehydrogenase and by mitochondrial swelling. MEHP may play an important role in the hepatotoxic effect associated with DEHP as well as its effect on mitochondria.

Rowland has pointed out the strain difference between Wistar and Sprague–Dawley rats on the metabolism of DEHP by the contents of alimentary tract [28]. However, such a marked difference between two strains was not found in our mitochondrial experiments with DBP or DEHP.

In comparative studies on the effects of the components of phthalic ester, phthalic acid inhibited the respiration to some extent, while 2-ethylhexanol was little effective. With regard to normal alcohols, it has been reported that alcohols from methyl to octyl inhibited the mitochondrial respiration proportionally to the length of the chains [29]. We have found also the similar result with alcohols from methyl to butyl.

From the viewpoint of structure-inhibitory effect relationships, the effect of positional isomers of DBP was compared. The effect of DBP (ortho-substituted ester) was the strongest, followed by that of di-*n*-butyl isophthalate (*meta*) and di-*n*-butyl terephthalate (*para*). Di-*n*-butyl maleate showed a remarkable effect in contrast to the corresponding fumarate. From these results, it was concluded that the *ortho* or *cis* form of dialkyl phthalate was more effective than the others. Furthermore, cyclohexane-1,2-dicarboxylate also gave the strong inhibitory effect, so it seemed that aromatic ring was not always essential to this kind of inhibition. A similar tendency was seen in both cyclohexane-1,2-dicarboxylates and phthalates with regard to the effect of alkyl groups on the respiratory inhibition.

Various inhibitors of respiration have been used to analyze the mechanism of oxidative phosphorylation [30]. Then, in order to elucidate the nature of respiratory inhibition by phthalates, DBP or MEHP was compared with several well known inhibitors. The inhibitory effects of these phthalates were observed mainly on the state-3 respiration and P:O ratio, and this fact suggests that phthalates have the property of energy-transfer inhibitor. When these phthalates were compared with oligomycin, a typical energy-transfer inhibitor, their inhibitions were somewhat different in the time course of inhibition, and entirely different in subsequent stimulation by DNP. Another difference between MEHP and oligomycin was observed in their effects on DNP-stimulated respiration. DBP showed the effect somewhat similar to

uncoupling agents, but was not so potent as DNP, a representative uncoupler.

When compared with oligomycin and DNP, it may be supposed that the blocking site of these phthalates lies before the DNP-sensitive site of respiratory chain. Superficially, the inhibitions of phthalates were different from those of malonate and rotenone, electron transfer inhibitors, while in higher concentration and prolonged reaction time, MEHP seemed to inhibit even the endogenous respiration. Consequently, the effect of phthalates would be also associated with the electron transfer inhibition.

With regard to the inhibitory effects of di-*n*-alkyl phthalates on the state-3 respiration, our present result was agreed with Ohyama's report except DPP was the most potent instead of DBP. On the other hand, the inhibition of the state-4 respiration was apparently different between the two authors. Ohyama showed the clear parallel relationship between per cent inhibition in state-3 and state-4 and the carbon number of the alkyl groups of phthalic esters, whereas we could find no inhibition of the state-4 by a series of dialkyl phthalates and rather stimulating effect of DBP. This discrepancy might be attributable to the difference of the amount of mitochondrial fraction added to the reaction mixture: there may have been differences in morphological purity, and the amount of protein added was different—2.0–2.8 mg in our case, and 1.0 mg in the work of Ohyama.

Recently we ascertained that the effect of DBP on the state-4 respiration was changed from stimulatory to inhibitory when the amount of mitochondrial protein was decreased stepwise from 4.5 mg to 1.0 mg. This fact shows that DBP acts as an uncoupler when the concentration of mitochondrial protein was high, but it acts reversely as an electron transport inhibitor when the concentration was low. However, real mechanism of the inhibition by phthalates is remained to be determined in future.

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