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Increase in hepatic ubiquinone on administration of diethylhexyl phthalate to the rat

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Abstract. It is shown for the first time that the content of ubiquinone of liver increases (2·5 fold) on dietary administration of the widely-used industrial plasticizer diethylhexyl phthalate to the rat. The increase is localized almost entirely in mitochondria in which the concentration of the quinone per mg protein is 1·7 times the control. Incorporation of the radioactive precursor (acetate) reveals that the biosynthesis of ubiquinone is increased in the livers of plasticizer-administered animals. The rate of degradation is not altered.

Keywords. Diethylhexyl phthalate; liver; ubiquinone; increase; synthesis.

Introduction

The plasticizer, di(2-ethylhexyl) phthalate (DEHP, also called dioctyl phthalate), is a major constituent of flexible plastics used in the packaging of food stuffs and in, the manufacture of medical devices. Since it is not convalently bound, the phthalate ester may easily leach out of the plastic. Thus, the compound has been detected in stored blood as well as in the tissues of patients receiving blood transfusion. Because of this and its occurrence ubiquitously in the soil, plant and stored food, the toxicity of the plasticizer has been investigated in detail (Thomas *et al.,* 1978). Administration of the plasticizer to experimental animals produces profound biochemical changes in the liver. Most of these changes resemble those produced by the antihypercholesterolemic drug Clofibrate. Recently, we have investigated the serum cholesterol-lowering property of DEHP first reported by Reddy *et al.* (1976) and have shown that the compound regulates the concentration of cholesterol in circulation both by inhibition of synthesis and by stimulation of degradation (Nair and Kurup, 1986).

Based on the observation that oral administration of ubiquinone effectively increases its concentration in the liver and also inhibits the synthesis of cholesterol *in vivo,* the hypothesis has been advanced that hypocholesterolemic agents exert their physiological action *via* ubiquinone (Krishnaiah, *et al.,* 1967a). Support for this view was provided by the fact that the lipid quinone branches from the bio synthetic, pathway of cholesterol (Ramasarma, 1972) and the observation that administration of Clofibrate increases the concentration of ubiquinone in liver (Krishnaiah *et al.*, 1967b; Philips *et al.,* 1968; Krishnaiah and Ramasarma, 1970; Kurup *et al.,* 1970; Krishnakantha and Kurup, 1974). Further, it was reported from our laboratory that the azodye 2-methyl-4-dimethyl-aminozobenzene depressed the concentration of cholesterol in circulation and raised the content of ubiquinone in the livers of rats (Saikumar and Kurup, 1984). It was of interest to see whether the correlation between serum cholesterol level and hepatic ubiquinone content holds good in the

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Abbreviations used: DEHP, Di(2-ethylhexyl) phthalate; HMGCoA, 3-hydroxy 3-methyl glutaryl coenzyme A.

case of DEHP also. The data presented in this paper reveal for the first time that administration of the plasticizer to the rat increases the concentration of ubiquinone in the liver and that the increase is achieved by a stimulation of the biosynthetic rate.

Materials and methods

Animals

Male albine rats (130-150 g) fed with a commercial Hind Lever diet (vegetable based; carbohydrate 53%, protein 25%, minerals 9%, fat 5% and fibre 4%) and kept in a room with artificial lighting (from 9·00 a.m. to 5·00 p.m.) were used in the experiments reported here. The phthalate ester was fed mixed in the diet powder (2% w/w) for the time period indicated.

Subcellular fractionation

Animals were' killed by cervical dislocation and hepatic mitochondria isolated by differential centrifugation (Kurup *et al.,* 1970). The particles were washed once with 0·25 M sucrose and used for estimation of ubiquinone. The microsomal fraction was sedimented by centrifugation at 39,000 *g* for 1 h.

Estimation of ubiquinone

The content of ubiquinone in liver tissue and subcellular fractions was determined by spectrophotometry after saponification, extraction and chromatography on deactivated alumina (Joshi *et al*., 1963). The concentration of the quinone was calculated from the decrease in absorbance at 340 nm of an ethanolic solution on reduction with $NaBH_4$ (Ramasarma, 1968). The content was also estimated by high performance liquid chromatography in a LKB unicord S II system using a Lichrosorb RP18 column (4× 250 nm) and ethanol as the eluent (flow rate 1 ml/min). Concentration was calculated from peak area using standard ubiquinone-9 (Abe *et al.,* 1981). In order that 'cyclic' variations (Edwin *et al.,* 1962) may not vitiate the results, an equal number of control and experimental animals were killed in the morning.

Turnover of ubiquinone

The rate of synthesis of ubiquinone was followed by determining the incorporation of [l-^{14}C]-acetate. The precursor was injected intra-peritoneally and the animals were killed 30 min later. To determine the decay rates of ubiquinone, rats were injected with the precursor and killed 1, 2 and 4 days later. The livers were processed for the isolation and estimation of ubiquinone.

Protein was estimated by the biuret method, deoxycholate being used for solubilization (Gornall *et al.,* 1949), Bovine serum albumin was used as standard. Samples for determination of radioactivity were dissolved in benzene (0·5 ml), added to vials containing 10 ml of 2,5-diphenyloxazole (0·5% w/v) in toluene and counted in a LKB Rack-Beta II Liquid Scientillation Counter. The background was deducted from all values.

Chemicals

The plasticizer was purchased from Solvents and Chemicals Co. Bangalore. Radioactive acetate and mevalonate were purchased from Bhabha Atomic Research Centre, Bombay. All biochemicals were obtained from Sigma Chemical Co., St. Louis, Missouri, USA. Other chemicals used were of the purest grades available. Solutions were prepared in water double-distilled in all-quartz apparatus and adjusted to the desired pH before use.

Results

Ubiquinone content of liver

Administration of the plasticizer results in an increase in the concentration of ubiquinone in the liver. Significant increase (30%; $P < 0{\cdot}05$) is observed in a week's time. In about 3 weeks the maximum increase (about 150%) is obtained (figure 1). The values for control animals agree closely with those reported in the literature (Ramasarma, 1985a). It may be pointed out that the increase obtained is of the same order (2-3-fold) as has been observed on administration of Clofibrate (Krishnakantha and Kurup, 1974).

It may be pertinent to mention in this context that the diet contained 8·8 ±1·4 nmol,

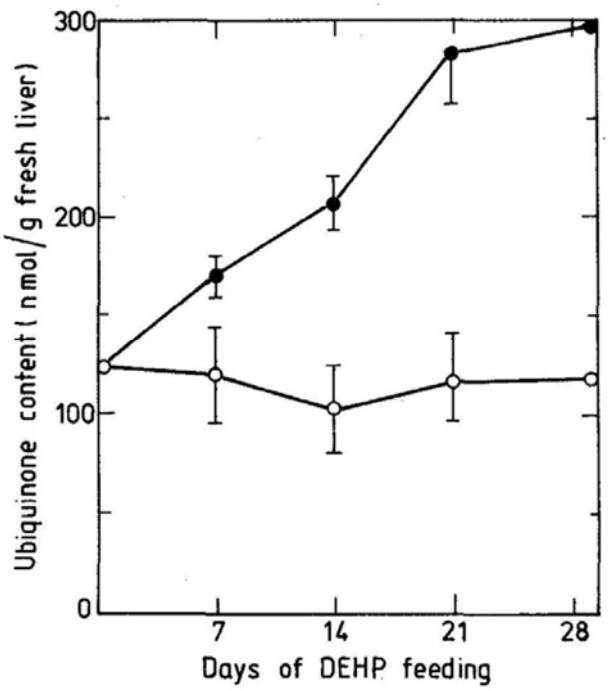

Figure 1. Effect of administration of diethylhexyl phthalate (DEHP) to the rat on the concentration of ubiquinone in liver. The concentration of ubiquinone (nmol) per g of liver in control (O) and DEHP-fed (2% w/w) in the diet (●)animals are given. Some typical values of standard deviation are also indicated. Experimental details are given in the 'materials and methods' section.

of ubiquinone/g. The average dietary consumption of ubiquinone in control and DEHP-fed animals was 146 and 149 nmol/rat per day respectively.

Ubiquinone synthesis

In order to gain an insight into the mechanism of increase of hepatic ubiquinone, the incorporation of acetate into the lipid quinone was studied. The results presented in table 1 reveal that administration of the plasticizer stimulates the synthesis of the quinone in liver. The incorporation of the precursor into the non-saponifiable lipid fraction per g of liver is decreased by almost 50% in DEHP-fed animals. This is consistent with our observation that in these animals both the specific activity of 3'-hydroxy 3-methyl glutaryl (HMGCoA) reductase (Mevolonate: NADP oxidoreductase (acylating CoA), EC 1.1.1.34) and the incorporation of acetate into hepatic cholesterol are inhibited by 50% (Nair and Kurup, 1986). Incorporation of the precursor into ubiquinone which is only a small fraction (4± 1%) of the total incorporation into the non-saponfiable lipids of whole liver in control animals, increases more than 5-fold (21 ± 8%) in the plasticizer-fed ones. The specific radioactivity of ubiquinone also is significantly (P <0.01) higher in the phthalate-ester-fed animals (2572±332 counts/min/μmol) than that in the control ones (1329 + 207 counts/min/μmol).

Table 1. Effect of administration of diethylhexyl phthalate on the synthesis of ubiquinone in rat liver.

	Counts/min per g liver	
Lipid fraction	Control	Plasticizer-fed
Non-saponifiable	5883 ± 800 (100)	3766 ± 448* (64)
Ubiquinone	221 ± 8 (4 ± 1)	839 ± 187* (14 ± 3)

Rats were injected with [1- ^{14}C] -acetate and killed 30 min later. The plasticizer was given in the diet (2% w/w) for 30 days. The values are the mean ± S.D. of 4 independent determinations (animals). The values in parentheses indicate the incorporation taking total incorporation into the non-saponfiable lipids of control animals as 100. *P<0·01 Control *vs* experimental.

Ubiquinone turnover

Determination of the rate of degradation of the quinone was done by measurement of the decay of radioactivity (Waterlow *et al*., 1978). The rate constant K_d was calculated from the equation

$$\ln \frac{A_0}{A_t} = K_d \cdot t,$$

Where A_o is the amount of lipid (radiolabel) at zero time and A_t at time 't'. The slope

of the plot of ln A_t against 't' yielded K_d. By definition, half-life is given by the equation

$$t_{\frac{1}{2}} = \frac{0{\cdot}693}{K_d}.$$

The half-life of ubiquinone calculated in this manner for control (2·6 ± 0·2 days) and plasticizer-fed (30 days) animals (2·5 ± 0·4 days) is the same. These values agree well with the half life (2-3 days) calculated from the data of Joshi and Ramasarma (1966).

Mitochondrial ubiquinone

A major portion of the ubiquinone in the cell is localized in the mitochondrial fraction. Administration of the plasticizer is known to cause enlargement of the liver (Warren *et al.*, 1982). In order to see whether the increase in the content of ubiquinone in liver reflects only the increase in the population of mitochondria or not, the concentration of the quinone in the mitochondrial fraction was determined. The data presented in table 2 show that the content of ubiquinone in the mitochondrial membrane (per mg protein) increases by more than 70% (P <0·01). Our values for control animals agree well with those reported in the literature (Sastry *et al.*, 1961; Krishnakantha and Kurup, 1974). Quite in agreement with previous reports (Sastry *et al.,* 1961; Ramasarma, 1985a), the mitochondrial fraction accounts for about half of the total cellular ubiquinone (table 2). The proportion increases by 78% in plasticizer-fed animals. It may be stated that the microsomal fraction did not show any significant difference in the content of ubiquinone between control (30±6nmol/ 100 mg protein) and phthalate ester-fed (23 ± 6 nmol/100 mg protein) animals.

Table 2. Effect of administration of diethylhexyl-phthalate on the content of ubiquinone in rat liver mitochondria.

Mitochondrial ubiquinone	Control	Plasticizer-fed (nmol)
Per 100 mg protein	162 ± 19	277 ± 24*
Per g liver	63 ± 7	223 ± 16*
	(53)	(75)
Per whole liver	386 ± 41	2325 ± 278*
	(53 ± 6)	(78 ± 8)

Rats were administered with DEHP in the diet (2% w/w) for 30 days. The values in parentheses represent the content of ubiquinone in the mitochondrial fraction taking the corresponding liver ubiquinone as 100. The values are the mean ± S.D. of 4 independent determinations (animals). *P<0·01 Control *vs* experimental.

The data presented in table 2 were obtained by determining the concentration of ubiquinone by the spectrophotometric method. To confirm that the large increase of ubiquinone in the mitochondrial fraction was not an artifact of interference by an ultraviolet-absorbing reducible substance produced by the ester, the quinone content of the samples was estimated also by high performance reverse phase liquid chro-

matography. The values obtained by the latter method were 92 ±6% of the values by the spectrophotometric method in control and 120 ± 6 % in plasticizer-fed animals.

Discussion

The data presented here which form the first report on the ability of DEHP to increase the concentration of hepatic ubiquinone in *vivo,* is consistent with the hypothesis (Ramasarma, 1967) that the modulation of HMGCoA reductase activity achieved by hypocholesterolemic agents is mediated by ubiquinone. We have observed that administration of plasticizer causes a substantial (50%) decrease in the specific activity of the enzyme in hepatic microsomes (Nair and Kurup, 1986).

It may appear paradoxical that when the activity of the rate-limiting enzyme is inhibited and the pool size of mevalonate is small, the synthesis of ubiquinone is enhanced. Similar results have been reported in cholesterol feeding also (Krishnaiah *et al.,* 1967a). It is generally believed that in rat liver the availability of mevalonate and not that of the ring precursor limits the biosynthetic rate of the quinine (Ramasarma, 1985b). Enhanced biosynthesis of ubiquinone from acetate is possible even when the rate-limiting enzyme is inhibited because the relative incorporation of acetate into ubiquinone is insignificantly small (about 4% of the non-saponifiable fraction) while the incorporation into cholesterol (90%) is very large. Moreover, the possible existence of a regulatory step in the conversion of squalene to lanosterol (Cenedella, 1980; Volpe and Obert, 1982) would favour channeling of the precursor into the quinone biosynthetic pathway.

Increasing the content of hepatic ubiquinone is a property which the plasticizer shares with Clofibrate. However the intra-cellular distribution of ubiquinone in the two cases is not similar. In clofibrate-fed animals the concentration of ubiquinone in the mitochondrial membrane (per mg protein) is not increased. The increase is substantial on the otherhand, in the nuclear fraction (Krishnakantha and Kurup, 1974). In the case of the plasticizer, the increase in ubiquinone appears to be accounted for exclusively by the mitochondrial fraction.

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