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Biochemical Changes in Liver of Rats Fed the Plasticizer Di (2-Ethylhexy) Phthalate

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Phthalate ester plasticizers induce a variety of biochemical changes in the tissues of experimental animals (PEAKALL 1975; LAWRENCE 1978; BELL 1982). In the present study, we fed rats a diet containing 1% di(2-ethylhexyl) phthalate (DEHP, W/W) for 3 weeks and then examined the activities of microsomal acylCoA:cholesterol acyltransferase (ACAT, EC 2.3.1.26), mitochondrial adenine nucleotide translocase, and cholesterol biosynthesis in their livers, in vitro. These studies demonstrate for the first time that DEHP feeding inhibits microsomal ACAT, the principle cholesterol esterifying enzyme in the liver. The microsomal component of hepatic cholesterolgenesis was also inhibited under these conditions. Adenine nucleotide translocase, an important mitochondrial anion translocator in the inner mitochondrial membrane, was not affected by DEHP feeding.

METHODS AND MATERIALS

Male rats (Upj:TUC (SD) spf, 250-275g) which were fed either a stock diet (Purina Chow) or the stock diet containing 1% DEHP (di[2-ethylhexyl] phthalate, Eastman Kodak, Rochester, N.Y.) (BELL & NAZIR 1976) were killed by decapitation between 9 a.m. and 10 a.m. and their livers were quickly excised.

The livers were rinsed in 0.9% NaCl solution and 20% homogenates were prepared in 0.1M phosphate buffer pH 7.4. The mitochondrial and microsomal fractions were prepared from the homogenates by differential centrifugation as previously described in detail elsewhere (BELL 1975).

Adenine nucleotide translocase (ADNT) was assayed in the "forward" direction by measuring the uptake (translocation) of [8-^{14}C] ADP ([8-^{14}C] adenosine 5^{1}-diphosphate, SA 57.3 Ci/mol, Amersham, Arlington Heights, IL) into mitochondria as previously detailed (BELL 1980). Basically the assay mixture consisted of 0.60±0.05 mg mitochondrial protein suspended in 1.0 ml of Tris-HCl buffer, pH 7.4. The mixture was preincubated for 1 minute at 37°C and the reaction was initiated by adding 71,000 dpm [^{14}C] ADP to yield a final concentration of 40μm ADP in a total volume of 1.05 ml. The reactions were terminated after 1 minute by the addition of 5mM atractyloside (an inhibitor of ADNT) and the mitochondria were reisolated, washed twice, digested with a tissue solubilizer (Protosol, New England Nuclear Corp., Boston, MA), and assayed for radioactivity by liquid scintillation counting (BELL 1980). Assays of mitochondria performed in the presence of atractyloside were

used to make corrections for nonspecific [^{14}C] ADP uptake (BELL 1980).

Cholesterol esterification by microsomal acylCoA:cholesterol acyltransferase (ACAT, EC 2.3.1.26) was assayed in a total volume of 345µl of 0.1M phosphate buffer, pH 7.4, containing 0.8-0.9 mg microsomal protein and 2.10^5 dpm [1-^{14}C] oleoylCoA (S A 59.7 Ci/mol, New England Nuclear Corp., Boston, MA) (BELL & HUBERT 1980a). Assays were performed in 13x125mm glass tubes at 37° for 5 minutes and were initiated by adding the [^{14}C] oleoylCoA in 20µl of sodium acetate buffer. Assays were terminated by the addition of 1.0 ml of methyl alcohol and the samples were extracted with $CHCl_3$-MeOH (2:1, V/V) as previously described (BELL & HUBERT 1980a). The lipid extracts were fractionated by thin-layer chromatography (TLC) to isolate the sterol esters which were scraped from the TLC plates and assayed for radioactivity (BELL & HUBERT 1980a).

Protein analyses on the mitochondria and microsomes were performed using the method of Lowry et al. (1951).

Liver slices were prepared and incubated at 37° for 90 minutes in 3.5 ml Krebs-Ringer bicarbonate buffer, pH 7.4, containing 2.0 µCi DL-[2-^{14}C] mevalonic acid, DBED salt (SA 50.1 Ci/mol, New England Nuclear Corp., Boston, MA) as previously described (BELL 1976). After incubation, the tissues were extracted with $CHCl_3$-MeOH (2:1, V/V) and fractionated by TLC to isolate the C_{27}-sterol band (cholesterol) which was identified by co-chromatography with authentic cholesterol (BELL 1976).

RESULTS AND DISCUSSION

AcylCoA:cholesterol acyltransferase (ACAT) is a microsomal enzyme responsible for the esterification of cholesterol by the liver (BELL & HUBERT 1980a). The data of Table 1 clearly show for the first time that DEHP feeding to rats results in a substantial reduction (70%, $p < 0.001$) in the activity of the enzyme. Figure 1 shows the kinetics of cholesterol esterification by ACAT in microsomes isolated from livers of DEHP-fed and control rats. The rate of cholesterol esterification is clearly reduced throughout the 0.25-5 minute assay period with DEHP-feeding. The inhibition of ACAT by DEHP-feeding does not represent a selective effect on microsomal ACAT since the biosynthesis of cholesterol from ^{14}C-mevalonate was also significantly inhibited (56%, $p < 0.02$) (Table 2). Whereas cholesterol esterification is accomplished by a single microsomal enzyme (ACAT), the post-mevalonate segment of the sterol biosynthetic pathway involves a variety of microsomal enzymes (SABINE 1977) with specific cofactor requirements. Cholesterol biosynthesis was consequently measured in liver slices. The inhibition of cholesterol

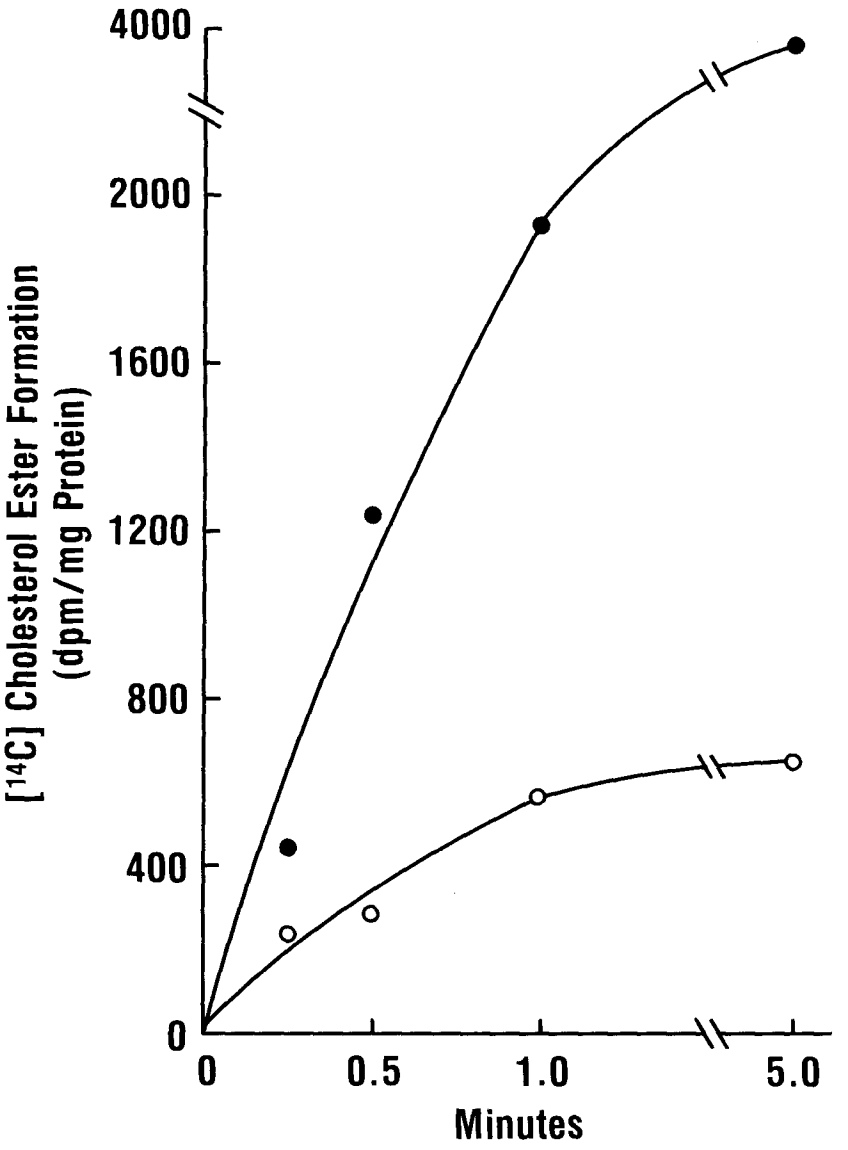

Figure 1. Kinetics of cholesterol esterification by ACAT in microsomes isolated from control and DEHP-fed rats. Microsomes isolated from livers of control and 1% DEHP-fed (3 wk) male rats were assayed for ACAT activity as described under Methods. Each datum point is the mean of 2 values; (control, ●; DEHP, O).

synthesis oberved in these studies confirms our earlier work with DEHP in the rat (BELL 1976) and considering the inhibition of cholesterol esterification (Table 1), illustrates the extent to which DEHP feeding modifies different aspects of sterol metabolism. Paradoxically, the inhibition of hepatic sterol synthesis does not result in a reduction of hepatic sterol levels (BELL 1976; BELL et al. 1978a) although it may account for the hypocholesterolemic effect of DEHP in rodents (REDDY et al. 1976; BELL et al. 1978a; 1978b).

TABLE 1. The effect of DEHP feeding on the activity of hepatic microsomal acylCoA:cholesterol acyltransferase in the rat.

	^{14}C-Oleoyl CoA Incorporation into Cholesterol Esters (dpm/mg protein)[a]	% Control
Control	5615 ± 510[b]	100
DEHP	1745 ± 390[c]	31

[a]ACAT assays using control microsomes contained 0.75 ± 0.08 mg protein/assay (n=5) and assay with microsomes from DEHP-fed animals contained 0.73 ± 0.06 mg protein per assay (n=5).

[b]Values are means ± SEM of data obtained from 5 animals per group. All assays were done in duplicate.

[c]Significantly different from control values ($p < 0.001$) using Student's Independent t-test.

TABLE 2. The effect of DEHP feeding on the biosynthesis of cholesterol from ^{14}C-mevalonate by rat liver slices.

	^{14}C-Cholesterol Synthesized[a] (dpm/g wet wt.)	% Control
Control	6930 ± 1205[b]	100
DEHP	3070 ± 520[c]	44

[a]Cholesterol biosynthesis was measured in liver slices from the animals using DL-[2-^{14}C]-mevalonic acid as a precursor.

[b]Values are means ± SEM of data obtained from 5 animals per group.

[c]Statistically different from control values ($p < 0.02$) using Student's Independent t-test.

In order to assess the functional properties of the liver mitochondria, we assayed adenine nucleotide translocase (ADNT). ADNT is one of the mitochondrial anion translocators and is located in the inner mitochondrial membrane (VIGNAIS 1976; BELL 1980). This enzyme plays a key role in energy metabolism since its function is to exchange (mol:mol) cytosolic ADP for intramitochondrially-generated

ATP, thus providing a flow of ATP into the cytosol to support cytosolic energy-requiring reactions (VIGNAIS 1976; BELL 1980). Results of the ADNT assays are presented in Table 3. Nucleotide transport activity in isolated mitochondria from livers of control and DEHP-fed rats had mean values of 265 and 275 pmoles/mg protein/minute, respectively, (Table 3) thus indicating that at least this important functional property of the mitochondria was unaffected by DEHP feeding. Heart mitochondria, which are known to accumulate DEHP in vivo (NAZIR et al. 1971), have also been shown to have normal ADNT activity when isolated from heart of rats fed DEHP (BELL & HUBERT 1980b). The failure of DEHP feeding to alter ADNT activity, but to stimulate β-oxidation of fatty acids in mitochondrial from rats fed 1% DEHP (BELL & GILLIES 1977) and to depress mitochondrial succinate dehydrogenase in the rat and ferret (LAKE et al. 1975, 1976) indicates that different functions of the mitochondria can be affected to differing extents by DEHP-feeding. It also seems clear that not all phthalate esters affect mitochondria in the same way. For instance, di-n-butyl phthalate is a potent inhibitor of state-3 respiration in isolated rat liver mitochondrial whereas DEHP has no effect (TAKAHASHI 1977).

The mechanism by which DEHP results in the inhibition of ACAT and cholesterol biosynthesis in rats is presently unknown. There is the possibility that changes in liver phospholipid synthesis induced by DEHP (BELL & NAZIR 1976; YANAGITA 1978) could alter the phospholipid composition of the endoplasmic reticulum and thereby alter membrane fluidity. Since ACAT and the microsomal enzymes of sterol synthesis are affected by changes in membrane fluidity (BELL & HUBERT 1980a; MITROPOULOS & VENKATESAN 1977), this possibility must be considered. Alternately, the insertion of a few molecules of DEHP into the endoplasmic reticulum might conceivably change the fluidity properties of the local lipid environment of enzymes and alter their reactivity (BELL 1982).

TABLE 3. The effect of DEHP feeding on the activity of adenine nucleotide translocase in isolated liver mitochondria

	^{14}C-ADP Translocation (pmoles/mg protein/min.)	% Control
Control	265 ± 15[a]	100
DEHP	275 ± 40[b]	104

[a]Values are means ± SEM of data from 5 animals per group. All assays were done in duplicate.

[b]Values not significantly different from the control values using Student's Independent *t*-test.

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