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Effect of DEHP on Adenine Nucleotide Translocase Activity in Isolated Rat Heart Mitochondria

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DEHP (di-2-ethylhexyl phthalate), a commonly used plasticizer in the production of PVC (polyvinylchloride) plastics, has been found to leach from PVC medical devices such as catheters and storage bags for blood and blood products (JAEGER & RUBIN 1970, 1972, MARCEL & NOEL 1970, EASTERLING *et al.* 1974, HILLMAN *et al.* 1975). As a result, patients undergoing medical treatment are frequently, but inadvertently exposed to DEHP. Of particular interest to us was the observation by HILLMAN *et al.* (1975) that infants undergoing transfusion and catheterization accumulated DEHP in heart tissue. In addition, NAZIR *et al.* (1971) reported the accumulation of DEHP in heart mitochondria from bovine, rabbit, rat, and dog and RUBIN & JAEGER (1973) reported that a level of DEHP attainable in stored blood (4 µg/ml) was lethal to cultured heart cells. These investigations prompted us to examine the possible effects of DEHP on the function of heart mitochondria and we subsequently reported that DEHP feeding to rats resulted in a transient inhibition of fatty acid oxidation by isolated heart mitochondria (BELL 1976, BELL & GILLIES 1977). In the present study, we have investigated the effect of DEHP and DEHP-feeding on the activity of adenine nucleotide translocase (AdNT) in isolated rat heart mitochondria.

MATERIALS AND METHODS

Male Sprague-Dawley rats (Upjohn:TUC (SD)spf) weighing 225-250 g were used in the studies. The rats were fed, ad libitum, with a stock chow diet (Purina Laboratory Chow) or the stock diet containing 0.5% DEHP (di-2-ethylhexyl phthalate, Eastman Kodak) by weight (BELL & NAZIR 1976). The animals were killed by decapitation between 9 AM and 10 AM and the hearts quickly excised and rinsed in chilled 0.9% NaCl. Heart mitochondria were isolated in 0.1 M phosphate buffer, pH 7.4, as previously described (BELL & GILLIES 1977) and resuspended in 40 mM Tris-HCl, 100 mM KCl, 1.0 mM $MgCl_2$ at pH 7.4 (SHUG *et al.* 1971) in readiness for enzyme assay. Adenine nucleotide translocase (AdNT) was assayed in the "forward" direction by measuring the uptake (translocation) of ^{14}C-ADP (8-^{14}C adenosine 5'-diphosphate, Amersham Corp.) into the mitochondria as described by WOJTCZAK & ZALUSKA (1967). 1.0 ml of mitochondrial suspension containing 0.59 ± 0.02 mg protein (mean ± SEM of 36 experiments) was preincubated for 1 min at 37°C. The assay was initiated by adding 20 µl of buffer containing 71000 dpm of ^{14}C-ADP to yield a final ADP concentration of

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40 µM. In incubations receiving DEHP, DEHP was added dissolved in 30 µl of acetone immediately prior to the preincubation period. The reactions were terminated after 60 seconds of incubation by addition of atractyloside (SHRAGO *et al.* 1974) to a final concentration of 5 mM. Samples preincubated for 1 min with atractyloside (5 mM) prior to additions of ^{14}C-ADP were used to correct the data for non-specific ^{14}C-ADP uptake (ca. 8-10% of total radioactivity taken up). The mitochondria were re-isolated by centrifugation at 10000 x g for 10 min at 5°C then washed twice by resuspension in 2.5 ml of the original suspending buffer. The washed mitochondrial pellets were dissolved in 1.0 ml of tissue solubilizer (Protosol, New England Nuclear Corp.) and then diluted with 15 ml of Liquifluor (New England Nuclear Corp.) for radioactive assay in a Packard Model 3375 liquid scintillation spectrometer. Quench corrections were made by the external standard method; counting efficiency was approx. 90%.

RESULTS AND DISCUSSION

Adenine nucleotide translocase (AdNT) is one of the mitochondrial anion translocators located in the inner mitochondrial membrane (KLINGENBERG *et al.* 1969, VIGNAIS 1976). The enzyme is of central importance in cellular metabolism because it catalyses a mole per mole exchange of extramitochondrial ADP for intramitochondrial ATP (KLINGENBERG *et al.* 1969, VIGNAIS 1976). It is through this exchange mechanism that mitochondrial ATP generated via oxidative phosphorylation is transported into the cytosol for cytosolic energy-requiring processes. Inhibition of the enzyme results in blockage of oxidative phosphorylation (VIGNAIS 1976). It is this latter point that suggested to us to investigate the effect of DEHP on AdNT since we previously reported that DEHP feeding to rats resulted in a transient inhibition of β-oxidation in heart mitochondria (BELL & GILLIES 1977).

Figure 1 shows the effect of DEHP on AdNT activity in isolated heart mitochondria from normal rats. DEHP was added to the mitochondrial incubations at levels yielding final concentrations of 5-600 µM. DEHP addition resulted in a decline in AdNT activity which reached a maximal inhibition of about 35% at 300-600 µM DEHP. At 100 µM, and less, inhibition was somewhat variable but nevertheless consistently below control values.

In order to investigate the effect of DEHP feeding on heart AdNT, the enzyme was assayed in heart mitochondria from rats fed DEHP for 3, 6, or 10 days (Table 1). DEHP feeding did not result in a statistically significant change in mitochondrial AdNT as was observed when it was added exogenously (Fig. 1). The results of Table 1 suggest that the decrease in fatty acid oxidation observed previously in heart mitochondria from DEHP-fed rats (BELL & GILLIES 1977) is unrelated to alterations in AdNT. The fact that DEHP affected AdNT when added directly to mitochondria (Fig. 1) but not when fed to the animals may indicate that the level of DEHP reaching the heart, or accumulating in the heart during the 3-10 day trials was insufficient to significantly alter AdNT.

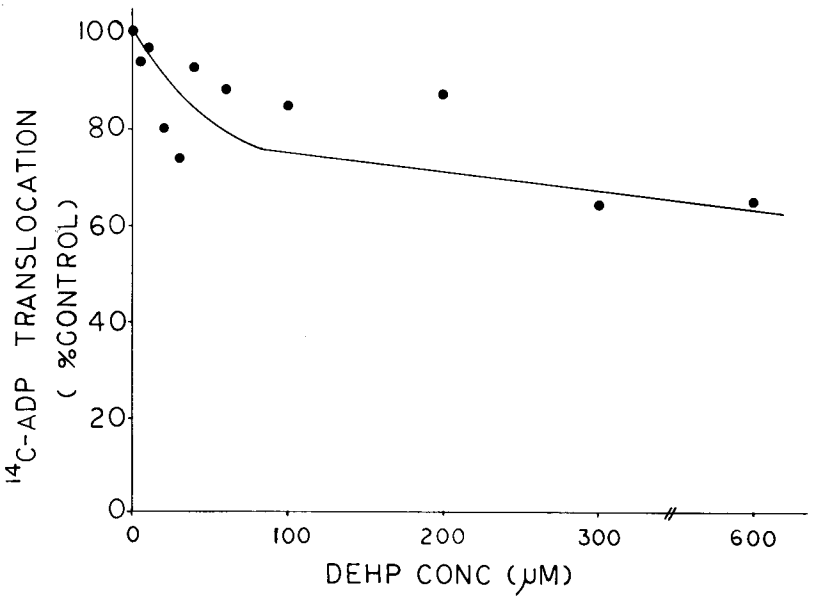

Figure 1. Inhibition of adenine nucleotide translocase in isolated rat heart mitochondria in the presence of various concentrations of DEHP. Enzyme assays were conducted as described under MATERIALS AND METHODS. All values represent the means of 3 to 4 individual animals except those values at 20, 40 and 60 μM DEHP which represent means of 15 animals each.

Although we did not measure DEHP levels in the heart of our animals, we know from previous studies that levels of 9 μg/g of heart have been measured in rats fed 0.1% DEHP in the diet (STEIN *et al.* 1974). In addition, studies on the subcellular distribution of DEHP in heart tissue from the bovine have reported that >99% of heart DEHP resides in the mitochondrial fraction (NAZIR *et al.* 1971). If such is also the case with other species, 1 ml of a 1 μM concentration of DEHP in the presence of 0.6 mg of mitochondrial protein as in our assay would be equivalent to 2.92 μg DEHP/g heart (based on our yields of approximately 4.5 mg mitochondrial protein/g heart tissue). By the same calculation, inhibition of AdNT at 50 μM DEHP would be equivalent to 146 μg DEHP/g heart. Interestingly, levels of 133 μg/g heart have been reported in rats after chronic inhalation of DEHP (GONZALEZ *et al.* 1976) and levels of 135 μg/g were reported in heart muscle from slaughter-beef whose source, or route of exposure was unknown (NAZIR *et al.* 1971). Our results, when considered with

TABLE 1. Adenine nucleotide translocase activity in mitochondria isolated from heart of rats fed DEHP for 3 to 10 days[a]

Duration of DEHP feeding (days)	^{14}C-ADP translocation pmol/mg protein/min
0	639 ± 46 (18)[b]
3	642 ± 83 (6)
6	669 ± 58 (6)
10	770 ± 89 (6)

[a]Adenine nucleotide translocase was assayed in heart mitochondria as described under MATERIALS AND METHODS.

[b]Values are means ± SEM of the number of animals designated by the values in parentheses.

the studies cited above indicate that mammalian heart can accumulate levels of DEHP that can be shown to inhibit heart AdNT, *in vitro*. Although it cannot be stated that similar affects could be expected *in vivo*, it is possible that inhibition of AdNT by DEHP could explain the toxicity of DEHP to cultured heart cells (RUBIN & JAEGER 1973) and in perfused heart systems (PETERSEN *et al.* 1975, LAWRENCE 1978). The effect of DEHP on heart muscle AdNT shown in our studies (Fig. 1) may be an important finding in view of the fact that human heart can accumulate DEHP (HILLMAN *et al.* 1975) as can heart tissue in non-human primates (JACOBSON *et al.* 1977). At the present time, we are unaware of data on DEHP levels in adult human heart and do not know to what levels DEHP could be expected to reach; levels of up to 5 µg/g of heart have been reported in infants, however (HILLMAN *et al.* 1975). Since DEHP is known to persist in the heart of man and non-human primates after cessation of obvious exposure (HILLMAN *et al.* 1975; JACOBSON *et al.* 1977) the possibility that cumulative effects of DEHP from a wide variety of sources could result in significant tissue levels has to be considered.

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