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Investigation of the Potential for Binding of Di(2-ethylhexyl) Phthalate (DEHP) to Rat Liver DNA *in Vivo*

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It was the aim of this investigation to determine whether or not covalent binding of di(2-ethylhexyl) phthalate (DEHP) to rat liver DNA could be a mechanism of action contributing to the observed induction of liver tumors after lifetime feeding of rodents with high doses of DEHP. DEHP radiolabeled in different positions was administered orally to female F344 rats with or without pretreatment for 4 weeks with 1% unlabeled DEHP in the diet. Liver DNA was isolated after 16 hr and analyzed for radioactivity. Administration of [^{14}C]carboxylate-labeled DEHP resulted in no measurable DNA radioactivity. With DEHP [^{14}C]- and [^{3}H]-labeled in the alcohol moiety as well as with 2-ethyl[1-^{14}C]hexanol, radioactivity was clearly measurable in the DNA. HPLC analysis of enzyme-degraded DNA revealed that the normal nucleosides had incorporated radiolabel whereas no radioactivity was detectable in those fractions where the carcinogen-modified nucleoside adducts are expected. A quantitative evaluation of the negative data in terms of a limit of detection for a covalent binding index (CBI) indicates that covalent interaction with DNA is highly unlikely to be the mode of tumorigenic action of DEHP in rodents.

Introduction

Covalent binding of reactive metabolites of organic chemicals to DNA in the target cell is an important early event in the carcinogenicity of a large number of carcinogens. This interaction can have a number of mutational consequences so that many short-term tests on mutagenicity can be used for a qualitative screening of chemicals for DNA interactions. The amount of carcinogen bound to DNA is dependent on a variety of parameters, such as the concentration at the site of metabolism, the rate of enzymatic activation and of enzymatic and nonenzymatic inactivation reactions, the diffusion to the critical DNA target, and the reactivity towards nucleic acids as opposed to other nucleophiles. Because of the complexity of these in part competing processes, only an *in vivo* situation is appropriate for a quantitative evaluation of the DNA damage in an attempt to estimate the carcinogenic potency of a chemical known to bind to DNA.

The binding of a test compound to DNA *in vivo* has been determined with the use of radiolabeled markers for about 100 chemicals (*1*). With the introduction (*2*) of the covalent binding index [CBI = (μmole chemical bound per mole DNA nucleotides)/(mmole chemical administered per kilogram body weight)] to normalize the DNA damage to the dose administered, a correlation of carcinogenic potency with CBI became possible and showed that a CBI of 10^4 to 10^3 can form the basis for a potent carcinogenicity, while a CBI of around 100 is found with moderate carcinogens and of 1 to 10 for weak carcinogens (*3*). This correlation also means that chemicals with a CBI of less than 0.1 are highly unlikely to give rise to a carcinogenicity detectable in a long-term bioassay unless they have, in addition to a minute DNA binding activity, some cocarcinogenic or promoting activity (*4*).

The possibility for such a distinction between DNA binding on the one hand and other mechanisms on the other hand in the process of tumor formation was the reason for the interest in performing DNA-binding assays *in vivo* with compounds which gave rise to an increased tumor incidence in a rodent bioassay. The idea behind it was the understanding that an extrapolation to low doses to which man is exposed will be linear for the formation of DNA–carcinogen adducts, while nonlinearities and possibly thresholds could be expected for the low-dose extrapolations with other mechanisms of carcinogenic action.

With the use of radiolabeled test compound for a DNA-binding study, only those nucleotide–carcinogen adducts are detectable which still carry the radiolabel. Since DEHP is composed of essentially two structural elements, the phthalic acid and the alcohol, differently marked DEHP molecules (I–IV) were used (Fig. 1).

The long-term exposure to DEHP used in the carcinogenicity studies could have induced qualitative and/or quantitative changes in the enzymatic drug metabolism pattern. In order to investigate whether such pretreatment could have an effect on DNA binding, one group of

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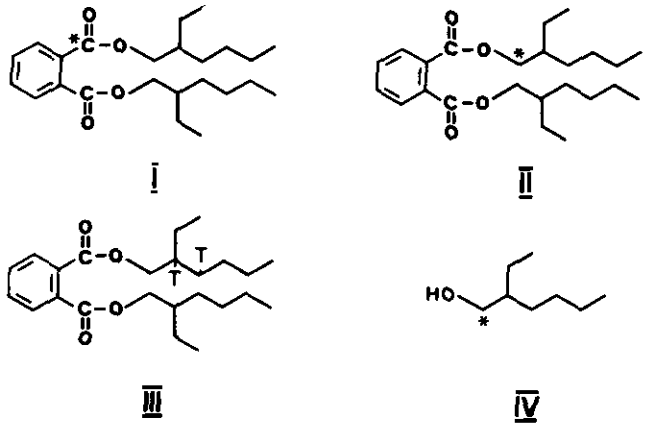

FIGURE 1. Chemical structures of test compounds: (I, II, III) di(2-ethylhexyl) phthalate (DEHP); (IV) 2-ethylhexanol (EH); (*) denotes ^{14}C; (T) denotes ^{3}H.

animals was pretreated for 4 weeks with a diet containing 10 g/kg DEHP (1%).

Methods and Materials

A detailed report on this study has recently been published (*5*). The following is only a brief overview.

Female F344 rats were from Charles River (Wiga, Sulzfeld, FRG) and weighed between 182 and 199 g at the time of treatment with the radiolabeled compounds. Duplicate animals were used for all experiments. DEHP was administered by oral gavage in olive oil on a chemical dose level of 500 mg/kg. The radiolabel dose was 10 mCi/kg [^{14}C]DEHP (I). DEHP II and III were administered together at 5 mCi [^{14}C] plus 40 mCi [^{3}H] per kg body weight. 2-Ethyl[1-^{14}C]hexanol (EH IV) was given on a dose level of 50 mg (about 14 mCi)/kg. The animals were placed in all-glass metabolism cages, and the carbon dioxide expired was collected for a determination of the metabolic stability of the [^{14}C]-label administered. After 16 hr, the animals were killed with ether, the liver was excised and homogenized immediately, and a crude chromatin fraction was prepared. DNA was purified by extractions of the chromatin, hydroxyapatite adsorption chromatography, dialysis, and precipitation with ethanol.

DNA was degraded with DNAase, phosphodiesterase, and alkaline phosphatase to the deoxyribonucleosides, or with hydrochloric acid to the purine bases and apurinic acid. Separation of the DNA constituents was performed with reverse-phase HPLC.

The specific activities determined for the DNA samples were expressed in the units of the covalent binding index (CBI), in order to allow a quantitative comparison with other carcinogens and noncarcinogens (*1*).

Results

Total DNA Radioactivity

After the administration of carboxylate-labeled DEHP I, a minute amount of radioactivity could be detected in only one out of four DNA samples. In all other samples, irrespective of the DEHP pretreatment, the radioactivity was below the limit of detection of the order of 0.02 CBI units. When the label was in the alcohol moiety (DEHP II and III), DNA radioactivity was easily detected in all cases, regardless of whether the label was ^{14}C or ^{3}H. Pretreatment with DEHP had no influence on total radioactivity in DNA after oral administration of either label. Apparent binding indices of 3 and 0.5 were calculated for the [^{14}C]- and [^{3}H]-labels, respectively. After administration of [^{14}C]EH IV, the radioactivity in the DNA was about twice the respective value obtained from [^{14}C]DEHP II administration, and apparent binding indices of about 6 resulted. This higher specific activity of DNA was accompanied by a higher fraction of the radioactivity dose expired in the form of CO_2.

Analysis of Nucleosides

An analysis of deoxyribonucleosides was carried out with the DNA obtained from rats treated with [^{14}C]- and [^{3}H]-labeled DEHP (II and III) and [^{14}C]-labeled EH IV. It could be shown that the normal nucleosides were radiolabeled, whereas no radiolabel was detectable in those fractions which normally contain the more lipophilic DNA–carcinogen adducts. After deduction of this anabolic incorporation of radiolabel from the original (total) specific activity of DNA, maximum possible CBI values of 0.1 to 0.5 and 0.6 to 0.9 resulted for DEHP II and EH IV, respectively, while the tritium data from DEHP III gave an upper bound of 0.2 to 0.3 for true covalent binding.

Another way of estimating maximum possible CBI could be based upon the general assumption that carcinogen–deoxyribonucleoside adducts are expected to elute after the normal constituents due to a higher lipophilicity of such adducts. No radioactivity could be detected in this region. On the basis of the standard deviation of the background radioactivity determined from a DNA sample isolated from an untreated animal, a limit of detection for a possible carcinogen-deoxyribonucleoside adduct was calculated. The maximum CBI of DEHP then was below 0.07 for [^{14}C]DEHP II, below 0.04 for [^{3}H]DEHP III and below 0.03 for [^{14}C]EH IV.

Analysis of Purines

The above discussion is based upon the general knowledge that nucleoside adducts of typical genotoxic carcinogens such as benzo(a)pyrene, 7,12-dimethylbenz(a)anthracene, or 3-methylcholanthrene elute after the natural nucleosides, due to increased lipophilicity. It is possible that smaller adducts such as methylated or ethylated nucleosides would not elute much later than the parent natural nucleoside. Therefore, a purine base analysis was performed after acid hydrolysis of two DNA samples of two animals treated with DEHP II plus III, one each with and without DEHP pretreatment. The HPLC system chosen was known to separate 7-methyl- or 7-ethylguanine, the most abundant alkylation products

in DNA, from their parent base. No radioactivity was detectable at elution volumes known for methylated or ethylated products. A limit of detection of CBI < 0.05 and < 0.09 resulted for [^{14}C]DEHP II and CBI < 0.03 and < 0.05 resulted for [^{3}H]DEHP III, with and without DEHP pretreatment, respectively.

Discussion

The results show that a covalent binding of DEHP to rat liver DNA must be below a CBI value of 0.05. This upper bound is 200,000 times below the CBI for the potent hepatocarcinogen aflatoxin B_1 and 20 to 200 times below the CBI of weakly DNA-binding carcinogens, where very large daily doses are required to produce an increase in tumor-bearing animals in a standard long-term bioassay.

The negative binding data derived from these experiments with DEHP therefore suggest that the tumorigenicity of this compound was probably due to a mode of action not related to DNA binding. In such a situation, tumor induction could go in parallel with some type of biological response—possibly species specific—which might also be observable in the long-term bioassay. Most of these biological responses are expected to follow the sigmoid dose-effect relationship generally known from other pharmacological activities. Such a mechanism might therefore allow the consideration of a threshold and it will be most important to search for other effects of DEHP on the animals' biology and find out whether the processes which parallel the induction of tumors in the bioassay are also found in humans exposed to much lower doses.

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