

## Investigation of the Potential for Binding of Di(2-ethylhexyl) Phthalate (DEHP) and Di(2-ethylhexyl) Adipate (DEHA) to Liver DNA *in Vivo*<sup>1</sup>

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Investigation of the Potential for Binding of Di(2-ethylhexyl) Phthalate (DEHP) and Di(2-ethylhexyl) Adipate (DEHA) to Liver DNA *in Vivo*. VON DÄNIKEN, A., LUTZ, W. K., JÄCKH, R., AND SCHLATTER, C. (1984). *Toxicol. Appl. Pharmacol.* 73, 373-387. It was the aim of this investigation to determine whether covalent binding of di(2-ethylhexyl) phthalate (DEHP) to rat liver DNA and of di(2-ethylhexyl) adipate (DEHA) to mouse liver DNA could be a mechanism of action contributing to the observed induction of liver tumors after lifetime feeding of the respective rodent species with high doses of DEHP and DEHA. For this purpose, DEHP and DEHA radiolabeled in different parts of the molecule were administered orally to female rats and mice, respectively, with or without pretreatment for 4 weeks with 1% unlabeled compound in the diet. Liver DNA was isolated after 16 hr and analyzed for radioactivity. The data were converted to a covalent binding index, CBI = (micromoles of substance bound per mole of DNA nucleotides)/(millimoles of substance applied per kilogram body weight), in order to allow a quantitative comparison also with other carcinogens and noncarcinogens. Administration of [<sup>14</sup>C]carboxylate-labeled DEHP to rats resulted in no measurable DNA radioactivity. The limit of detection, CBI < 0.02 was about 100 times below the CBI of compounds where an observable tumor-inducing potential could be due to genotoxicity. With [<sup>14</sup>C]- and [<sup>3</sup>H]DEHP labeled in the alcohol moiety, radioactivity was clearly measurable in rat liver DNA. HPLC analysis of enzyme-degraded or acid-hydrolyzed DNA revealed that the natural nucleosides or purine bases were radiolabeled whereas no radioactivity was detectable in those fractions where the carcinogen-modified nucleoside or base adducts are expected. The respective limits of detection were at 0.07 and 0.04 CBI units for the <sup>14</sup>C and <sup>3</sup>H labels, respectively. The experiments with [<sup>14</sup>C]- and [<sup>3</sup>H]DEHA, labeled in the alcohol moiety and administered to mice, revealed a minute radioactivity of <50 dpm/mg liver DNA, too little to allow a nucleoside analysis to determine that fraction of the radioactivity which had been incorporated via biosynthesis. Expressed in the CBI units, values of 0.05 to 0.15 for <sup>14</sup>C and 0.01 to 0.12 for <sup>3</sup>H resulted. Determination of the level of <sup>14</sup>CO<sub>2</sub> expiration revealed a linear correlation with the specific activity of DNA. Experiments with 2-ethyl[1-<sup>14</sup>C]hexanol performed with both rats and mice allowed the conclusion that most if not all DEHA radioactivity in mouse liver DNA was due to biosynthetic incorporation. A maximum possible true DNA binding by DEHA must be below CBI 0.01. Pretreatment of the animals with unlabeled compound had no effect on the DNA radioactivities in either species. The present negative data, in conjunction with other negative short-term tests for mutagenicity, strongly indicate that covalent interaction with DNA is highly unlikely to be the mode of tumorigenic action of DEHP and DEHA in rodents.

The plasticizer di(2-ethylhexyl) phthalate (DEHP) was reported to induce liver tumors in F344 rats and B6C3F1 mice in a 2-year

of the Federation of the Chemical Industry (CEFIC) on behalf of the Bureaux Internationaux Techniques (BIT), Plasticiser Toxicology and Ecology Committee.

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feeding experiment (National Toxicology Program, 1982a). The doses used (12,000 and 6000 ppm DEHP in the diet for the rats and 6000 and 3000 ppm for the mice) caused increased incidences of hepatocellular carcinoma or neoplastic nodules in female rats of either dose group and in male rats of the higher dose group as well as hepatocellular carcinoma and adenoma in mice of either sex and dose group.

Di(2-ethylhexyl) adipate (DEHA) was reported to induce liver tumors in mice in a 2-year feeding experiment with 12,000 ppm and 25,000 ppm DEHA admixed to the diet (National Toxicology Program, 1982b). Increased incidences of hepatocellular adenoma and carcinoma in males and females were found in either dose group. In rats, no difference in incidence or type of tumors between treated and control animals was observed after a 2-year feeding with 12,000 or 25,000 ppm DEHA in the diet.

Considering the widespread use of DEHP and DEHA and the possible exposure of the general population to low doses, it seems important to determine whether the tumor-inducing potential of these compounds is based upon a genotoxic mode of action via DNA binding or whether other mechanisms are responsible. The answer is essential for any extrapolation between species and from high to low doses.

DEHP and DEHA are inducers of peroxisomes in rats (Moody and Reddy, 1978; Reddy, 1981; Mangham *et al.*, 1981; Gray *et al.*, 1982). The peroxisome inducers may form a novel class of carcinogens (Reddy *et al.*, 1980). On the other hand, data on a putative interaction of radiolabeled DEHP with rat liver DNA *in vivo* have been presented by Albro *et al.* (1982). In a subsequent report (Albro *et al.*, 1983) which included data on dual label experiments, this group presented indirect evidence that the association of radiolabel with DNA might have been due to biosynthetic incorporation. The main limiting factor in their studies was the fact that the low level of DNA radioactivity allowed no analysis

of the DNA constituents to distinguish between biosynthetic incorporation of the radiolabel and true covalent deoxyribonucleotide-DEHP adducts.

These preliminary findings prompted us to perform a DNA-binding assay with DEHP in rat and mouse liver and DEHA in mouse liver. Large amounts of radioactivity were administered in our study in order to achieve sufficiently high specific activities in the DNA to allow for an analysis of the source of the radiolabel and to improve the limit of detection in case of a negative result. The present study was carried out with DEHP and DEHA radioactively labeled in three different positions as illustrated in Fig. 1. A  $^{14}\text{C}$  label was introduced either at the carboxylate carbon atom of the phthalate moiety (I) or at C-1 of the alcohol moiety of DEHP (II) and of DEHA (V).  $^3\text{H}$ DEHP II and  $^3\text{H}$ DEHA VI were tritiated in positions 2 and 3 of the alcohol moiety. In order to investigate whether 2-ethylhexanol (EH), a known metabolite of DEHP and DEHA, can interact with DNA,  $[1-^{14}\text{C}]$ EH IV was also studied.

The long-term exposure to DEHP and DEHA used in the carcinogenicity studies could have induced qualitative and/or quan-

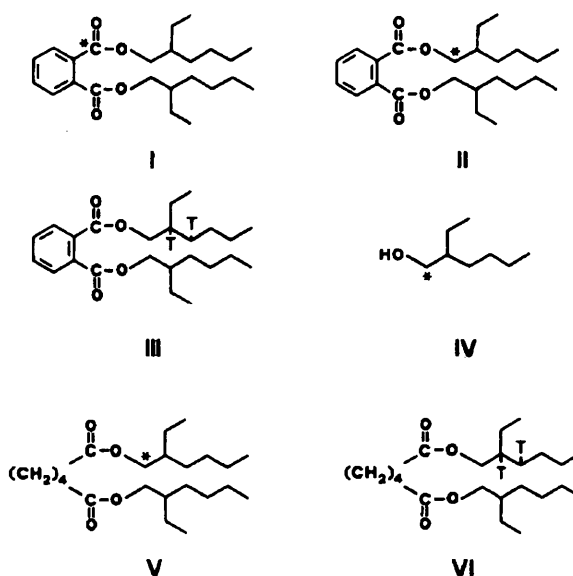


FIG. 1. Structures of radiolabeled test compounds used. (I, II, III) Di(2-ethylhexyl) phthalate; (IV) 2-ethylhexanol; (V, VI) di(2-ethylhexyl) adipate. \* =  $^{14}\text{C}$ , T =  $^3\text{H}$ .

titative changes in the enzymatic drug metabolism pattern. In order to investigate whether such pretreatment could have an effect on DNA binding, groups of animals were pretreated for 4 weeks with a diet containing 10 g/kg DEHP or DEHA.

## METHODS

**Chemicals and radiolabeled compounds.** All enzymes were obtained from Sigma Chemical Company, St. Louis, Missouri. DEHP,  $^{14}\text{C}$ -labeled in the carboxylate carbon (Fig. 1, compound I) was synthesized at ICI, Macclesfield, UK, with a specific activity of 46.8 mCi/mmol. DEHP and DEHA labeled with  $^{14}\text{C}$  and  $^3\text{H}$  in the alcohol moiety (II, III, V, and VI) were synthesized at BASF AG, Ludwigshafen, FRG, with specific activities of 39.5, 132.8, 38.9, and 124.7 mCi/mmol, respectively. The [ $^{14}\text{C}$ ]EH IV was produced at BASF with a specific activity of 34.4 mCi/mmol. The radiochemical purity was checked 1 day before use by thin-layer chromatography on silica-gel plates with methylene chloride for DEHA and DEHP and methylene chloride:methanol 1:1 for EH. The radiochemical purity was 97.8% for [ $^3\text{H}$ ]DEHA and >99% for the other compounds. Unlabeled DEHP was obtained from Fluka AG, Buchs, Switzerland. The compounds were dissolved in olive oil (Konsum Verein, Zurich, Switzerland). One milliliter of the application solution of [ $^{14}\text{C}$ ]DEHP I contained 136 mg DEHP with 2.54 mCi. Compounds II and III were administered together to rats as a solution of 129 mg DEHP/ml with 1.12 mCi/ml of [ $^{14}\text{C}$ ]DEHP and 9.86 mCi/ml of [ $^3\text{H}$ ]DEHP. Compounds V and VI were administered together to mice as a solution of 119 mg DEHA/ml with 3.85 mCi/ml of [ $^{14}\text{C}$ ]DEHA and 27.7 mCi/ml of [ $^3\text{H}$ ]DEHA. The  $^{14}\text{C}$  to  $^3\text{H}$  ratio was chosen to be about 1:8 because of different counting efficiencies and the  $^{14}\text{C}$  spillover in the  $^3\text{H}$  channel which reduces the accuracy of the determination of  $^3\text{H}$  counts. [ $^{14}\text{C}$ ]DEHP II was administered to mice as a solution of 26.4 mg DEHP/ml olive oil with 2.67 mCi/ml of  $^{14}\text{C}$  label. EH IV was used undiluted. One milliliter of the application solution contained 12.0 and 11.0 mg EH with 3.15 and 2.91 mCi  $^{14}\text{C}$  for the treatment of rats and mice, respectively.

**Animals and treatments.** Female rats of the Fischer 344 strain and female mice of the NMRI strain, 150–200 and 21–23 g body wt, respectively, were supplied by Charles River Wiga, Sulzfeld, FRG. They were held in Macrolone cages in groups of four animals and were marked by tail painting. Room temperature of  $21 \pm 2^\circ\text{C}$  and humidity of  $55 \pm 15\%$  were registered continuously. The illumination time was 12 hr per day. The animals were fed with diet No. 343 purchased from Kliba Klingentalermühle, Kaiseraugst, Switzerland (Batch Nos. 55/81 and 59/82). Analysis of contaminants of feed was performed by RCC, Itingen, Switzerland, and conformed to EPA specifications

of maximally tolerable levels. The pretreated groups were fed a diet containing 10 g/kg DEHP and DEHA, respectively, prepared and analyzed with respect to concentration, stability, and homogeneity by BASF. Feed and water were given *ad libitum*. Feed consumption was measured daily. The body weight was monitored weekly.

After 2 to 3 weeks of acclimatization, one group of animals was given the DEHP- or DEHA-containing diet for 4 weeks while the others received the regular chow. The radiolabeled test compounds were then administered by oral gavage of the solutions described above on dose levels indicated in the tables. For each experiment two animals served as unlabeled controls and were treated with no radioactivity at all.

**Pharmacokinetics.** After the administration of the radiolabeled compounds, the animals were held in all-glass metabolism cages, and urine was collected. An air stream of 0.4 liter/min transported the expired air to a trap with ethanolamine:methanol 1:4 to collect the  $^{14}\text{CO}_2$  expired. The production of tritiated water, HTO, from [ $^3\text{H}$ ]DEHP was determined on the basis of the specific activity of the HTO in the urine after dilution with water and repeated recondensation.

**Isolation of DNA.** Sixteen hours after the administration of the radiolabeled compounds, the animals were killed by open heart puncture under ether anesthesia, and livers were excised and weighed. A crude chromatin fraction was prepared in the cold by a slightly modified method described by Yaneva and Dessev (1976): The liver was homogenized in 75 mM NaCl, 10 mM EDTA, 10 mM Tris/HCl, pH 7.8, in a Potter-Elvehjem homogenizer. Nonidet P-40 detergent was added to a concentration of 0.2%. After 15 min, the samples were centrifuged for 5 min at 700g. The crude chromatin pellet was washed once with 10 mM EDTA, 10 mM Tris/HCl, pH 7.8. The isolation and purification of DNA from chromatin at room temperature followed the standard method used in our laboratory, which is based on a method of two extractions with phenol:chloroform:isoamyl alcohol 25:24:1 and a hydroxylapatite column chromatography as the main purification step (Markov and Ivanov, 1974). After dialysis against water at  $4^\circ\text{C}$  overnight, addition of NaCl to a concentration of 0.2 M and precipitation after addition of a twofold volume of ethanol, the DNA was dried on an aspirator vacuum and dissolved in 4 or 5 ml of 10 mM Tris/HCl, 10 mM  $\text{MgCl}_2$ , pH 7.0. In the experiment with mice the ethanol precipitation step was repeated once. The DNA content was determined on the basis of a uv absorbance of 20 at 260 nm for a solution of 1 mg/ml. An aliquot of 1 ml was used for radioactivity counting. If no radioactivity was measurable, the remaining DNA solution was added to the vial and counted again. If radioactivity was detectable on a level of at least 50 cpm/mg, DNA was degraded enzymatically or deproteinized with hydrochloric acid, and the deoxyribonucleosides or purine bases were separated by HPLC to determine biosynthetic incorporation of radioactivity into the DNA.

*Determination of covalent binding indices (CBI).* The specific radioactivity of DNA was normalized by the dose administered:

$$CBI' = \frac{\text{dpm/mg DNA}}{\text{dpm/kg body wt}}$$

This value was converted to the molar units,

$$CBI = \frac{\mu\text{mol chemical bound/mol DNA nucleotide}}{\text{mmol chemical applied/kg body wt}}$$

according to  $CBI = CBI' \times 309 \times 10^6$  on the basis of an average molecular weight of 309 g/mol DNA nucleotides (Lutz, 1979).

*Analysis of deoxyribonucleosides.* DNA was degraded enzymatically to deoxyribonucleosides by a method slightly modified from King *et al.* (1977): 1–2 mg of DNA dissolved in 1 ml 10 mM Tris/HCl, 10 mM MgCl<sub>2</sub>, pH 7.0, was incubated for 16 hr at 37°C with 250 units of deoxyribonuclease I (EC 3.1.21.1) from bovine pancreas. Two hundred fifty microliters with 0.075 unit venom phosphodiesterase I (EC 3.1.4.1) and 2.5 units of alkaline phosphatase III (EC 3.1.3.1) from *Escherichia coli* in a solution of 50 mM Tris/HCl, pH 9.0, were added and incubated for 24 hr at 37°C. After filtration through 0.22- $\mu\text{m}$  filters (Millipore, Bedford, Mass.), the samples were loaded onto a HPLC reverse-phase column (LiChrosorb RP 18, 8  $\times$  250 mm, Knauer, Oberursel, FRG) and eluted with a flow of 3.5 ml/min for 56 min with a methanol:water gradient 0–10% for the first 5 min, 10–100% from 10 to 50 min. The optical density at 254 nm of the eluate was continuously recorded in a flow photometer (Perkin-Elmer LC55). Fractions of 7 ml were collected (unless indicated otherwise in Fig. 7) and added to 10 ml Insta-Gel (Packard) for radioactivity counting.

The background radioactivity in the <sup>14</sup>C channel was determined for each chromatogram on the basis of mean and standard deviation of fractions 1 and 11–28. The background radioactivity in the <sup>3</sup>H channel is not constant over the entire chromatogram due to the quenching by a higher methanol content in the later fractions. The background radioactivity of the first 12 fractions and the respective standard deviation were therefore determined with linear regression of the cpm values of fractions 1, 3, 4, 8, 9, 11, and 12. The background radioactivity of fractions 13–28 was determined on the basis of the mean and standard deviation of these single fractions obtained from control DNA.

*Analysis of purine bases.* The DNA was depurinated by acid hydrolysis. HCl (1 M) was added to the DNA solution to give a concentration of 0.1 M. The samples were incubated for 1 hr at 70°C (Meier-Bratschi *et al.*, 1983). After the samples were filtered through 0.22- $\mu\text{m}$  filters, they were loaded onto an HPLC reverse-phase column,  $\mu\text{Bondapak C}_{18}$  (7.8  $\times$  300 mm, Waters), and eluted with 3.5 ml/min 0.01 M ammonium phosphate, pH 4, with 1% methanol. The elution was monitored at 254 nm. Two-minute fractions of 7 ml were collected and added to 10 ml Insta-Gel for radioactivity counting.

The background radioactivity in the <sup>14</sup>C and <sup>3</sup>H channels was determined for each chromatogram on the basis of the mean and standard deviation of fractions 1, 4, 5, 7, 8, and 10–27.

*Scintillation counting: Statistics.* The radioactivity counting was carried out in a liquid scintillation counter, Tri-Carb 460 CD (Packard), with Insta-Gel (Packard) as the scintillation mixture. For small amounts of radioactivity, the samples were counted 4  $\times$  40 min. The background was determined as the mean count of two DNA samples isolated together with the test samples from animals which had not received radiolabels but which were held together with the test animals. These values, given in the tables and figures, varied between the experiments with standard deviations of  $\pm 1.1$  cpm and  $\pm 0.8$  cpm in the <sup>14</sup>C and <sup>3</sup>H channels, respectively (statistical mean of standard deviations within each pair of background counts) (Kaiser, 1965). This standard deviation was also used to decide whether significant radioactivity was detected in the vial containing DNA of treated animals. The counting efficiencies varied with the quench grade of the samples and were determined with internal standards. They are given in footnotes to the tables.

## RESULTS

### *General Observations and Pharmacokinetic Parameters*

*In the rat.* Feed consumption did not differ between the pretreated and the nonpretreated rats. The daily feed intake in the respective groups was  $12.2 \pm 1.2$  and  $13.8 \pm 2.1$  g per rat, resulting in a DEHP dose for the pretreated rats of  $0.65 \pm 0.05$  g/kg body wt per day. No deviation from the normal body weight gain known for this strain was observed.

The pretreatment of rats with DEHP in the feed (10 g/kg) for 4 weeks increased the liver weight to body weight ratio significantly, a finding which is in accordance with other reports (Carpenter *et al.*, 1953; Harris *et al.*, 1956; Moody and Reddy, 1978). It was  $56.2 \pm 2.4$  g/kg body wt compared to  $41.3 \pm 3.9$  g/kg body wt of the nonpretreated rats. The liver enlargement resulted in a more rapid metabolism of the subsequent dose of radio-labeled DEHP as is illustrated by the more rapid and more extensive exhalation of <sup>14</sup>CO<sub>2</sub> generated by the degradation of <sup>14</sup>C-labeled DEHP I and II (see below).

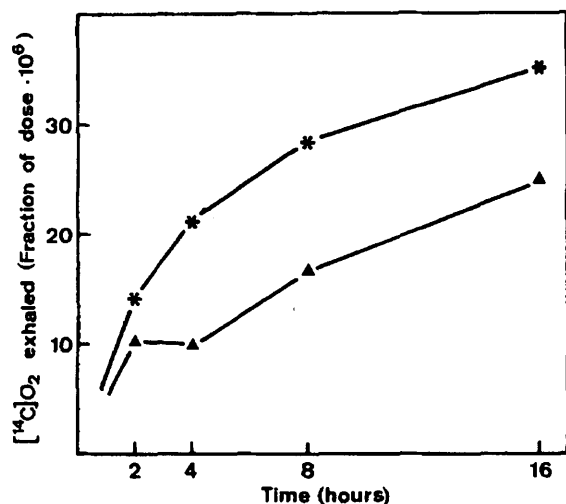


FIG. 2. Cumulative exhalation of  $^{14}\text{CO}_2$  after oral administration of  $[^{14}\text{C}]\text{DEHP I}$  to DEHP-pretreated (\*) and nonpretreated ( $\Delta$ ) rats. Means of two animals.

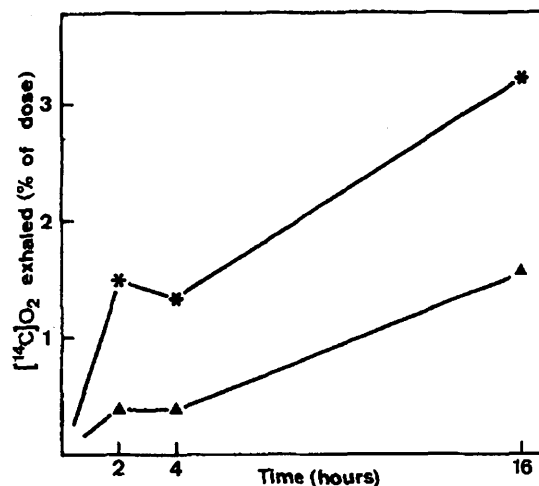


FIG. 3. Cumulative exhalation of  $^{14}\text{CO}_2$  after oral administration of  $[^{14}\text{C}]\text{DEHP II}$  to DEHP-pretreated (\*) and nonpretreated ( $\Delta$ ) rats. Means of two animals.

About one-third of the radioactive dose of all four compounds was excreted within 16 hr in the urine. By taking into account the additional metabolites still present in the body, it is reasonable to assume that at least half the dose had been absorbed from the gastrointestinal tract and passed the liver.

Exhalation of  $^{14}\text{CO}_2$  by the rats treated with the *carboxylate*-labeled DEHP I accumulated within 16 hr to less than a fraction of  $3 \times 10^{-5}$  of the radioactivity administered (Fig. 2). This finding is so small that it does not have to be the result of a metabolic cleavage of the carboxylate carbon of DEHP I from its benzene moiety, but could be due to radiolabeled impurities. The  $^{14}\text{CO}_2$  exhalation after administration of *alcohol*-labeled DEHP II is, however, in the order of a few percent of the dose (Fig. 3). DEHP pretreatment resulted in an increased production of  $^{14}\text{CO}_2$ . After administration of 1- $^{14}\text{C}$ -labeled 2-ethylhexanol IV, the fraction of expired  $^{14}\text{CO}_2$  represented as much as 15% (Fig. 4).

*In the mouse.* Feed consumption was slightly but not significantly increased in DEHA-pretreated mice. The daily feed intake in the respective groups was  $7.12 \pm 1.75$  and  $5.44 \pm 0.84$  g per mouse, resulting in a DEHA dose for the pretreated mice of  $2.09 \pm 0.57$  g/kg body wt per day. No deviation from the

normal body weight gain known for this strain was observed.

The DEHA radioactivity excreted in the urine within 16 hr was  $60 \pm 29$  and  $57 \pm 28\%$  of the  $^{14}\text{C}$  and  $^3\text{H}$  doses administered. By taking into account the additional metabolites still present in the body, it is reasonable to assume that most of the radiolabeled dose had been absorbed from the gastrointestinal tract and had passed the liver.

Production of tritiated water (HTO) from

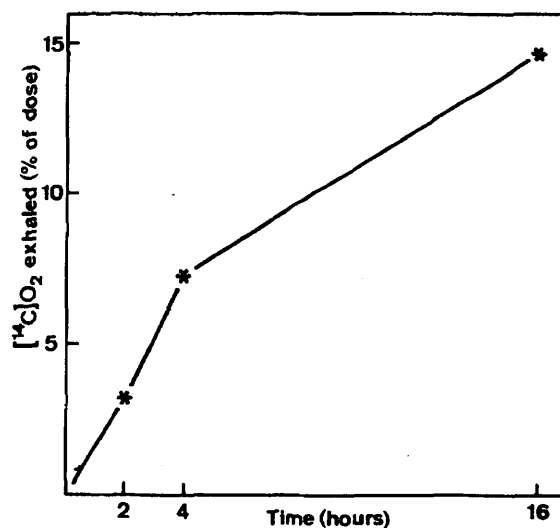


FIG. 4. Cumulative exhalation of  $^{14}\text{CO}_2$  after oral administration of  $[^{14}\text{C}]\text{EH IV}$  to DEHP-pretreated rats. Means of two animals.

[ $^3\text{H}$ ]DEHA was determined on the basis of the specific activity of HTO in the urine, which was between 1 and  $4 \times 10^7$  dpm/ml. Under the assumption that HTO is distributed homogeneously in the body fluid and that the total water content of a mouse is 60% of its body weight, 3 and 1.5% of the dose had been released as HTO in pretreated and nonpretreated animals, respectively.

The  $^{14}\text{CO}_2$  exhalation after administration of  $^{14}\text{C}$ -labeled DEHA, DEHP, and EH was in the order of a few percent of the dose. DEHA pretreatment resulted in an increased production of  $^{14}\text{CO}_2$ . The exhalation of  $^{14}\text{CO}_2$  after administration of [ $^{14}\text{C}$ ]EH was more rapid than that of DEHA and was more extensive (Fig. 5).

The pretreatment of mice with DEHA in the food (10 g/kg) for 4 weeks increased the liver weight to body weight ratio slightly but not significantly. It was  $78 \pm 24$  g/kg body wt as compared to  $61 \pm 14$  g/kg body wt of the nonpretreated mice. The more rapid metabolism of the radiolabeled DEHA, illustrated by an increased exhalation of  $^{14}\text{CO}_2$ , a more rapid excretion of radiolabel in the urine, and an increased HTO production, is probably due to this liver enlargement.

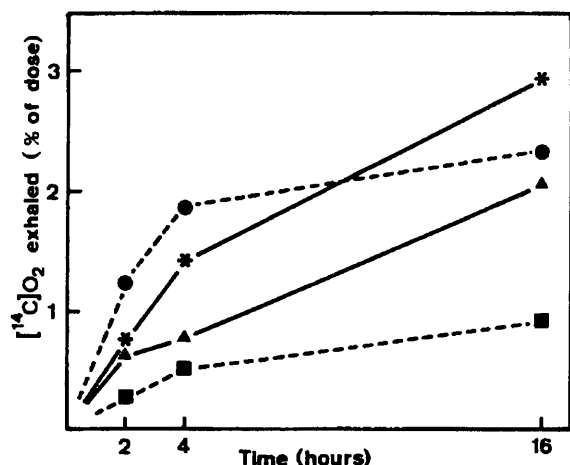


FIG. 5. Cumulative exhalation of  $^{14}\text{CO}_2$  after oral administration of [ $^{14}\text{C}$ ]DEHA I to DEHA-pretreated (\*) and nonpretreated (▲) mice, of [ $^{14}\text{C}$ ]EH IV (●) to DEHA-pretreated mice, and of [ $^{14}\text{C}$ ]DEHP III (■) to nonpretreated mice (dashed lines). Means of two animals.

#### DNA-Binding Studies in the Rat

The data on the radioactivity determined in the DNA are summarized in Table 1. With carboxylate-labeled DEHP I, minute radioactivity could be detected in only one 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 unit. In one case, a small amount of radioactivity was detected in the DNA. It is very unlikely that this radioactivity originated from a radioactive labeled DNA-phthalate adduct. Probably it is noncovalently bound radioactivity from contaminating proteins or labeled metabolites which may not have been completely removed in this specific sample. Our negative finding is in accordance qualitatively with that of Albro *et al.* (1982) who also did not find any radioactivity in the DNA after oral administration of carboxylate-labeled [ $^{14}\text{C}$ ]DEHP. Due to the fact that they administered only about  $6.6 \times 10^8$  dpm/kg, i.e., about 20 times less than the dose used in our study, their finding is less valuable, however, on a quantitative basis for an exclusion of a genotoxic activity.

When the label was in the alcohol moiety (DEHP II and III), radioactivity was easily detected in all samples, regardless of whether the label was  $^{14}\text{C}$  or  $^3\text{H}$ . Apparent binding indices of 3 and 0.5 were obtained for the  $^{14}\text{C}$  and  $^3\text{H}$  labels, respectively, and pretreatment was without influence. After administration of [ $^{14}\text{C}$ ]EH IV, the radioactivity in the DNA was about twice the respective value obtained from [ $^{14}\text{C}$ ]DEHP II administration and apparent binding indices of about 6 resulted.

The radioactivity associated with the DNA samples could be due to noncovalent interaction of the compound or some metabolite with DNA, to biosynthetic incorporation of radiolabeled precursors of DNA synthesis, or to true covalent adducts with DNA. Contamination of the DNA with radiolabeled protein cannot contribute substantially to the DNA radioactivity because the specific activity of chromatin protein was only about 50 times

TABLE I

RADIOACTIVITY IN FEMALE RAT LIVER DNA, 16 hr AFTER ORAL ADMINISTRATION OF DI(2-ETHYLHEXYL) [carboxylate-<sup>14</sup>C]PHTHALATE (I), DI(2-ETHYL[1-<sup>14</sup>C]HEXYL) PHTHALATE (II), DI(2-ETHYL[2,3-<sup>3</sup>H]HEXYL) PHTHALATE (III), AND 2-ETHYL[1-<sup>14</sup>C]HEXANOL (IV) TO DEHP-PRETREATED AND NONPRETREATED RATS, EXPRESSED PER UNIT DOSE

| Test compound:                              | [ <sup>14</sup> C]DEHP (I) |        | [ <sup>14</sup> C]DEHP (II) + [ <sup>3</sup> H]DEHP (III) |        |                   |       | [ <sup>14</sup> C]JEH (IV) |       |                   |       |
|---|----------------------------|--------|---|--------|-------------------|-------|----------------------------|-------|-------------------|-------|
|   | +                          | +      | -   | -      | +                 | +     | -                          | +     |                   |       |
| Chemical dose administered (mg/kg)          | 545                        | 520    | 528   | 558    | 530               | 524   | 501                        | 539   | 51.1              | 53.4  |
| Radioactive dose ( $\times 10^{10}$ dpm/kg) |                            |        |   |        |                   |       |                            |       |                   |       |
| <sup>14</sup> C                             | 2.27                       | 2.16   | 2.19  | 2.32   | 1.03              | 1.01  | 0.969                      | 1.04  | 2.99              | 3.12  |
| <sup>3</sup> H                              |                            |        |   |        | 9.00              | 8.91  | 8.51                       | 9.15  |                   |       |
| DNA in vial (mg)                            | 5.23                       | 6.24   | 5.66  | 5.53   | 1.28              | 1.22  | 1.15                       | 1.39  | 1.15              | 1.17  |
| Total count (cpm)                           |                            |        |   |        |                   |       |                            |       |                   |       |
| <sup>14</sup> C                             | 21.5                       | 18.3   | 29.0  | 17.5   | 110.0             | 114.1 | 109.1                      | 114.1 | 529.5             | 518.0 |
| <sup>3</sup> H                              |                            |        |   |        | 107.2             | 106.6 | 101.8                      | 108.8 |                   |       |
| Radioactivity in DNA <sup>a</sup>           |                            |        |   |        |                   |       |                            |       |                   |       |
| <sup>14</sup> C                             | <0.043 <sup>b</sup>        | <0.027 | 0.039 $\pm$ 0.026   | <0.026 | 2.94 <sup>c</sup> | 3.26  | 3.45                       | 2.79  | 6.06 <sup>d</sup> | 5.58  |
| <sup>3</sup> H                              |                            |        |   |        | 0.48              | 0.50  | 0.52                       | 0.45  |                   |       |

<sup>a</sup> The radioactivity is expressed in CBI units.

<sup>b</sup> In the experiment with compound I, the calculation of net radioactivity is based on background activities of 18.6 and 16.0 cpm for liver DNA isolated from two untreated animals. The background count was  $17.3 \pm 1.1$  cpm. Standard deviations and limits of detection are calculated on a level of  $p = 0.01$ . The counting efficiency was 74.0%.

<sup>c</sup> In the experiment with compounds II and III, the calculation of net radioactivity is based on background activities of 14.6 and 13.9 cpm in the <sup>14</sup>C channel and 26.2 and 26.8 cpm in the <sup>3</sup>H channel for liver DNA isolated from two untreated animals. Counting efficiencies were 76.4% for <sup>14</sup>C in the <sup>14</sup>C channel, 15.5% for <sup>14</sup>C in the <sup>3</sup>H channel, and 33.8% for <sup>3</sup>H in the <sup>3</sup>H channel. The <sup>3</sup>H counts in the <sup>14</sup>C channel could be neglected.

<sup>d</sup> In the experiment with compound IV, the calculation of net radioactivity is based on background activities of 13.6 and 13.7 cpm for liver DNA isolated from two untreated animals. The counting efficiency was 76.4%.

higher than that of DNA which is known to be contaminated by a maximum of 0.2% protein (Sagelsdorff *et al.*, 1983).

The relatively high  $^{14}\text{C}$  production seen after administration of DEHP II and EH IV indicates that a large fraction of the alcohol moiety is degraded in the body. The degradation products may then be used as precursors in the biosynthesis of the deoxyribonucleosides. After oral administration of [ $^{14}\text{C}$ ]methanol to rats, the  $^{14}\text{C}$  exhalation amounted to 80% of the dose. The radioactivity in the liver DNA isolated 12 hr after the administration of [ $^{14}\text{C}$ ]methanol corresponded to a CBI of 170 (Däniken *et al.*, 1981). The  $^{14}\text{C}$  exhaled after [ $^{14}\text{C}$ ]DEHP administration was 25–50 times smaller, in the range of 1.5–3% of the dose. A CBI in the region of 3–6 may therefore easily be explained on the basis of biosynthetic incorporation. This finding is in perfect agreement with the values around 3 as measured here (Table 1).

The  $^3\text{H}$  radioactivity in the DNA measured after the administration of [ $^3\text{H}$ ]DEHP III was about 12 times lower than the  $^{14}\text{C}$  radioactivity originating from DEHP II. Again, this radioactivity was shown to result primarily from biosynthetic incorporation into DNA of tritiated breakdown products produced during the oxidation of the labeled hexanol. Tritiated water was also shown to be formed and the specific activity of HTO in the 16-hr urine was about  $2 \times 10^7$  dpm/ml. Control experiments with oral administration of HTO and isolation of liver DNA after 24 hr revealed that radiolabel is associated with the DNA on an apparent CBI level of 0.5 (Lutz, 1979). This radioactivity is not lost from the DNA upon washing so that it does not represent simple proton–tritium ion exchange. One possibility for the formation of a stable carbon–tritium bond is given for instance by the reduction of the 2'-hydroxyl group of the ribonucleoside diphosphate to the 2'-deoxy derivative of DNA. A quantitative comparison similar to the one calculated above with the  $^{14}\text{C}$  label is rendered more complicated, however, because the HTO formed in the liver

cell from oxidative metabolism of DEHP III reaches a higher intracellular concentration as compared with HTO distributed uniformly after oral administration.

Although the above discussion clearly indicates that  $^{14}\text{C}$  and  $^3\text{H}$  radioactivities in the DNA isolated after administration of alcohol-labeled DEHP II and III can be explained by reasons other than covalent interaction of reactive metabolites, this hypothesis had to be corroborated on the basis of an analysis of DNA constituents.

*Analysis of nucleosides.* An analysis of deoxyribonucleosides was carried out with the DNA obtained from rats treated with  $^{14}\text{C}$ - and  $^3\text{H}$ -labeled DEHP II and III and  $^{14}\text{C}$ -labeled EH IV. The elution of the natural deoxyribonucleosides localized by their optical density at 254 nm was the same for all DNA samples and is shown on the bottom of Figs. 6 and 7. Retention times of 8.5 min for deoxycytidine (dC), 11.5 min for deoxyguanosine (dG), 13.5 min for thymidine (T), and 18 min for deoxyadenosine (dA) were recorded. The peak appearing at 10.0 min represented deoxyinosine (dI) which was formed in variable amounts during the hydrolysis of the DNA by enzymatic deamination of deoxyadenosine by enzyme impurities.

Figure 6 shows the elution of the  $^{14}\text{C}$  and  $^3\text{H}$  radioactivity of the digested DNA of animals that had been given alcohol-labeled DEHP (II plus III). It can readily be seen that no radioactivity elutes after deoxyadenosine, i.e., in the region known to contain the more lipophilic deoxyribonucleoside–carcinogen adducts, derived for example from benzo[*a*]pyrene (Jennette *et al.*, 1977; Boroujerdi *et al.*, 1981), dimethylbenz[*a*]anthracene (Jeffrey *et al.*, 1976), or 3-methylcholanthrene (King *et al.*, 1977). Radioactivity eluted exclusively with the front peak and with natural nucleosides. The fractions containing the natural nucleosides amounted to the largest part of the total radioactivity eluted, representing 87 to 97%  $^{14}\text{C}$  and 42 to 69%  $^3\text{H}$ . If these radioactivities, clearly due to biosynthetic incorporation of radiolabeled DEHP-breakdown



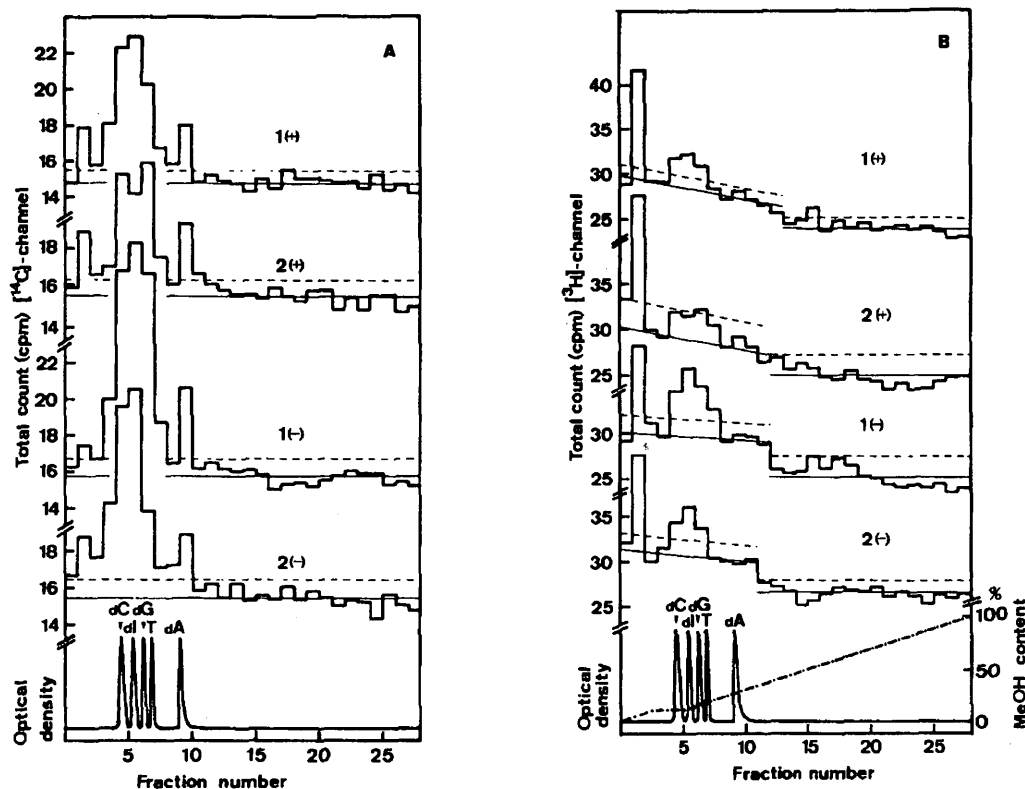


FIG. 6. HPLC elution profiles of deoxyribonucleosides obtained from enzymatically degraded liver DNA. Four rats had been orally administered DEHP (II plus III),  $^{14}\text{C}$ - and  $^3\text{H}$ -labeled in the alcohol moiety, two with (+) and two without (-) pretreatment with unlabeled DEHP. The optical density profile (254 nm; bottom diagram) was identical for all four samples. The solid line indicates the level of background radioactivity; the dashed line shows the limit of detection of the radioactivity in the different chromatograms. (A)  $^{14}\text{C}$  channel; (B)  $^3\text{H}$  channel.

products, are deducted from the total apparent CBI as given in Table 1, residual values of 0.1 to 0.5 and 0.2 to 0.3 for  $^{14}\text{C}$  and  $^3\text{H}$ , respectively, result. This view is the most conservative approach based on the assumption that the front peak is due to some sort of DNA adduct formed by the alcohol moiety of DEHP.

These front fractions, No. 2 plus No. 3 of the deoxyribonucleoside chromatograms, contained 3–13 and 31–58% of the  $^{14}\text{C}$  and  $^3\text{H}$  radioactivity, respectively. Such early eluting radioactivity is generally observed in HPLC analyses of DNA degradation products. It could be due to tightly but noncovalently bound DEHP metabolites, including tritiated water, released from the DNA only after breakdown to the nucleosides. It is also possible that it contains oligo-nucleotides formed

by incomplete enzymatic degradation, or sugars produced from degradation of apurinic acid. The latter DNA fragments could have their radioactivity from either biosynthetic incorporation or covalently bound test substance.

The same situation was observed with [ $^{14}\text{C}$ ]ethylhexanol IV (Fig. 7). After deduction of the biosynthetic incorporation of radiolabel (83 to 90% of the total specific activity), a maximum possible CBI of 0.6 to 0.9 could result. In this experiment all deoxyadenosine had been converted to deoxyinosine.

Another way of estimating maximum possible CBI could be based upon the general assumption that carcinogen–deoxyribonucleoside adducts are expected to elute only after 20 min in the region of fractions 11–28, due to a higher lipophilicity of such adducts. No

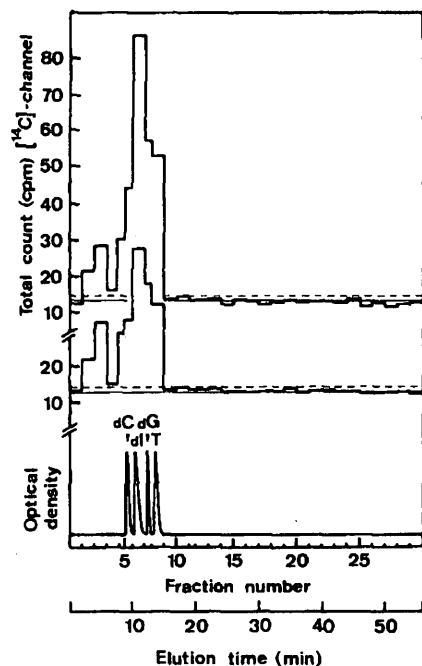


FIG. 7. HPLC elution profile of deoxyribonucleosides obtained from enzymatically degraded liver DNA. Two rats had been orally administered [ $^{14}\text{C}$ ]EH IV, after pretreatment with unlabeled DEHP. The optical density profile (254 nm; bottom diagram) was identical for the two samples. The solid line indicates the level of background radioactivity; the dashed line shows the limit of detection of the radioactivity in the different chromatograms.

radioactivity could be detected in this region. On the basis of the standard deviation of the background radioactivity (dashed line in Figs. 6 and 7), a limit of detection for a possible carcinogen-deoxyribonucleoside adduct can be given. The maximum CBI of DEHP is then below 0.07 for [ $^{14}\text{C}$ ]DEHP II, below 0.04 for [ $^3\text{H}$ ]DEHP III, and below 0.03 for [ $^{14}\text{C}$ ]EH IV. This limit of detection is therefore almost as low as that of the carboxylate-labeled DEHP I.

**Analysis of purines.** The above discussion is based upon the general knowledge that nucleoside adducts of typical genotoxic carcinogens such as benzo[*a*]pyrene, 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. The elution profile is shown in Fig. 8. No radioactivity was detectable at elution volumes known for methylated or ethylated products and a limit of detection was CBI < 0.05 and < 0.09 for [ $^{14}\text{C}$ ]DEHP II and CBI < 0.03 and < 0.05 for [ $^3\text{H}$ ]DEHP III, with and without DEHP pretreatment, respectively.

#### DNA-Binding Studies in the Mouse

The data on the radioactivity determined in the mouse liver DNA are summarized in Table 2. With [ $^{14}\text{C}$ ]DEHA V and [ $^3\text{H}$ ]DEHA VI, DNA radioactivity was detectable in all

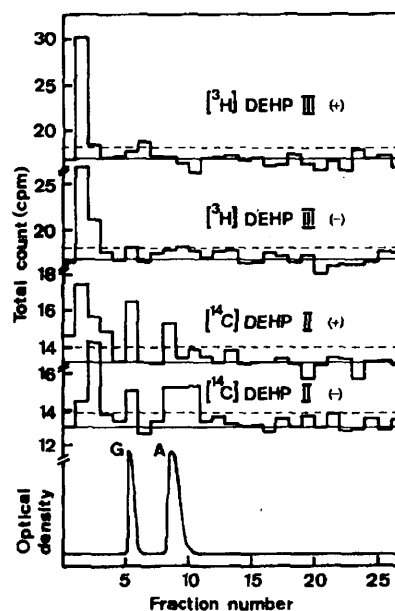


FIG. 8. HPLC elution profile of purine bases obtained from acid-hydrolyzed liver DNA. Rats have been orally administered [ $^{14}\text{C}$ ]- and [ $^3\text{H}$ ]DEHP (II plus III), one with (+) and one without (-) pretreatment with unlabeled DEHP. The optical density profile (254 nm; bottom diagram) was identical for the two samples. The solid line indicates the level of background radioactivity; the dashed line shows the limit of detection of the radioactivity in the different chromatograms.

TABLE 2

RADIOACTIVITY IN FEMALE MOUSE LIVER DNA, 16 hr AFTER ORAL ADMINISTRATION OF Di(2-ETHYL[1-<sup>14</sup>C]HEXYL) ADIPATE (V) AND Di(2-ETHYL[2,3-<sup>3</sup>H]HEXYL) ADIPATE (VI) TO DEHA-PRETREATED AND NONPRETREATED MICE, OF Di(2-ETHYL[1-<sup>14</sup>C]HEXYL) PHTHALATE (II) TO NONPRETREATED MICE, AND OF 2-ETHYL[1-<sup>14</sup>C]HEXANOL (IV) TO DEHA-PRETREATED MICE, EXPRESSED PER UNIT DOSE

| Test compound:                    | [ <sup>14</sup> C]DEHA (V) + [ <sup>3</sup> H]DEHA (VI) |                       | [ <sup>14</sup> C]DEHP (II) |                       | [ <sup>14</sup> C]EH (IV) |                       |                       |
|-----------------------------------|---|-----------------------|-----------------------------|-----------------------|---------------------------|-----------------------|-----------------------|
|                                   | +   | +                     | -                           | -                     | -                         | +                     |                       |
| DEHA pretreatment:                | +   | +                     | -                           | -                     | -                         | +                     | +                     |
| Chemical dose (mg/kg body wt)     | 1100  | 1050                  | 1260                        | 1440                  | 272                       | 297                   | 120.2                 |
| Radioactive dose (dpm/kg body wt) |   |                       |                             |                       |                           |                       |                       |
| <sup>14</sup> C                   | $7.89 \times 10^{10}$                                   | $7.57 \times 10^{10}$ | $9.09 \times 10^{10}$       | $1.03 \times 10^{11}$ | $6.09 \times 10^{10}$     | $6.66 \times 10^{10}$ | $7.07 \times 10^{10}$ |
| <sup>3</sup> H                    | $5.66 \times 10^{11}$                                   | $5.43 \times 10^{11}$ | $6.52 \times 10^{11}$       | $7.40 \times 10^{11}$ |                           |                       | $6.45 \times 10^{10}$ |
| DNA in vial (mg)                  | 0.47  | 0.50                  | 0.19                        | 0.37                  | 0.25 <sup>a</sup>         | 0.37                  | 0.35                  |
| Total count (cpm)                 |   |                       |                             |                       |                           |                       |                       |
| <sup>14</sup> C                   | 19.0  | 26.9                  | 15.2                        | 17.3                  | 13.8                      | 16.3                  | 34.4                  |
| <sup>3</sup> H                    | 22.5  | 26.4                  | 31.2                        | 19.6                  |                           |                       | 25.3                  |
| Radioactivity in DNA <sup>b</sup> |   |                       |                             |                       |                           |                       |                       |
| <sup>14</sup> C                   | 0.069 <sup>c</sup>                                      | 0.154                 | 0.057                       | 0.049                 | $0.035 \pm 0.029^d$       | $0.065 \pm 0.018$     | 0.37                  |
| <sup>3</sup> H                    | 0.022   | 0.030                 | 0.122                       | 0.013                 |                           |                       | 0.21                  |

<sup>a</sup> Precipitated only once with ethanol because of low yield.

<sup>b</sup> The radioactivity in the DNA is expressed in CBI units.

<sup>c</sup> In the experiment with compounds V and VI, the calculation of net radioactivity is based on background activities of 12.2 and 13.5 cpm in the <sup>14</sup>C channel and 15.5 and 14.9 cpm in the <sup>3</sup>H channel for liver DNA samples isolated from two untreated animals. Counting efficiencies were 73.8% for <sup>14</sup>C in the <sup>14</sup>C channel, 16.1% for <sup>14</sup>C in the <sup>3</sup>H channel, and 31.2% for <sup>3</sup>H in the <sup>3</sup>H channel. The <sup>3</sup>H counts in the <sup>14</sup>C channel could be neglected.

<sup>d</sup> In the experiment with compounds II and IV, the calculation of net radioactivity is based on background activities of 12.4 and 12.5 cpm for liver DNA samples isolated from two untreated animals. The background count was  $12.5 \pm 0.4$  cpm. The counting efficiency was 73.9%. Standard deviations calculated on a level of  $p = 0.01$  are shown in those cases where the net radioactivity was near the limit of detection.

samples. Upon comparison of individual values, the  $^3\text{H}$  value of one nonpretreated animal was almost ten times higher than that of the other. It is likely that this vial contained traces of  $^3\text{H}$  radioactivity not due to the DNA sample because the  $^{14}\text{C}$  levels were similar, and the  $^{14}\text{C}$  to  $^3\text{H}$  ratio of all other DNA samples was between 3 and 5 whereas that specific DNA sample showed a ratio of 0.5. By not taking into consideration this probably contaminated tritium value, apparent binding indices in CBI units of 0.05–0.15 and 0.01–0.03 resulted for the  $^{14}\text{C}$  and  $^3\text{H}$  label, respectively.

Although radioactivity in the DNA was detectable in all experiments, the specific activities were minute (<50 dpm/mg DNA for  $^3\text{H}$  and <38 dpm/mg for  $^{14}\text{C}$ ) after administration of extremely large doses of radioactivity ( $>5 \times 10^{11}$  and  $>7 \times 10^{10}$  dpm/kg body wt, respectively). It was therefore useless to degrade the DNA to nucleosides or bases because the HPLC analysis would not have allowed the determination of the fraction of radioactivity which was incorporated into DNA via biosynthesis. The only possibility for a quantitative estimation of that source of radioactivity was by measuring the formation of  $^{14}\text{CO}_2$  and HTO. The  $\text{CO}_2$  data compiled in Fig. 5 show that the DNA radioactivities were proportional to the initial rate of expiration of  $^{14}\text{C}$  label. This finding is a good indication that biosynthetic incorporation of radiolabeled breakdown products was the main reason for the DNA labeling.

The experiment with alcohol-labeled [ $^{14}\text{C}$ ]DEHP II in the mouse was performed to establish a link to the results obtained with rats. There, the radioactivity in the liver DNA expressed in CBI units was about 3 as opposed to the mean value of 0.05 for mouse (Tables 1 and 2). The difference by a factor of about 60 is due first to a species difference of about one order of magnitude for biosynthetic incorporation of  $^{14}\text{CO}_2$  into liver DNA as found earlier in control experiments with oral administration of [ $^{14}\text{C}$ ]methanol to mice and rats (data not shown), and second, to the fact that

the production of  $^{14}\text{CO}_2$  was about twice as high in the rat. The specific activity of the liver DNA isolated from the rats was high enough to allow proof that the natural deoxyribonucleosides were radiolabeled (Fig. 6). The DNA radioactivity measurable on the liver DNA of mice treated with [ $^{14}\text{C}$ ]DEHP II is therefore most likely also due to biosynthetic incorporation of radiolabeled breakdown products into DNA. The same argument holds for the results obtained after administration of [ $^{14}\text{C}$ ]EH IV.

The tritium radioactivity in the DNA measured after the administration of [ $^3\text{H}$ ]DEHA VI was three to five times lower than the  $^{14}\text{C}$  radioactivity originating from DEHA V if the specific activities are normalized to the dose administered by using CBI units. Again, the tritium radioactivity could result from biosynthetic incorporation into DNA of tritiated breakdown products, produced during the oxidation of the labeled hexanol. Tritiated water was shown to be formed and the specific activity of HTO in urine was between 1 and  $4 \times 10^7$  dpm/ml. This level could be achieved from an oxidation and release of 1 to 4% of the  $^3\text{H}$  activity administered. This fraction is in good agreement with the fraction of the  $^{14}\text{C}$  radioactivity expired in the form of  $\text{CO}_2$ . Control experiments with oral administration of HTO to mice and isolation of liver DNA after 24 hr revealed that radiolabel is indeed incorporated in the natural deoxyribonucleosides on an apparent CBI of 0.05 (Sagelsdorff, 1982). A quantitative comparison similar to the one performed above with the  $^{14}\text{C}$  label is rendered more complicated, however, because the HTO formed in the liver cell from oxidative metabolism of DEHA II will reach a higher intracellular concentration compared to HTO distributed uniformly after oral administration.

If all the evidence presented above is taken into account, there is little doubt that negligible or none of the DNA radioactivity detected after administration of [ $^{14}\text{C}$ ]- and [ $^3\text{H}$ ]DEHA can be due to covalent interactions. Any true

covalent binding of DEHA to mouse liver DNA will therefore be below  $CBI < 0.01$ .

## DISCUSSION

Our data do not give any indication for a covalent binding of DEHP and DEHA to liver DNA of rats and mice, respectively. Nucleoside and base analyses as well as correlations obtained between  $^{14}CO_2$  expired and specific activity of DNA, in both rats and mice, allowed us to conclude that the radioactivity associated with the DNA was largely due to biosynthetic incorporation of radiolabeled breakdown products. [ $^{14}C$ ]EH administration gave rise to the highest DNA radioactivities and to the highest level of  $^{14}CO_2$  exhalation. This result is in contrast to the data reported by Albro *et al.* (1982), where only RNA but not DNA was found to be radiolabeled after administration of [ $^{14}C$ ]EH. Although the amount of radioactivity administered was much smaller than in our experiments, it should have been possible to measure radioactivity in the DNA because Albro *et al.* (1982) did detect radioactivity in the DNA after a similar radioactive dose of [ $^{14}C$ ]DEHP II. Furthermore, in an additional experiment with mono(2-ethyl[1- $^{14}C$ ]hexyl) phthalate, they did measure radioactivity in both types of nucleic acids and the radioactivity in RNA was very similar to the value measured after administration of [ $^{14}C$ ]EH. This contrast is not readily explained since both nucleic acids contain the same nucleophilic centers, and the biosynthetic incorporation into RNA is unlikely to change so drastically from one experiment to another. We cannot explain their findings.

The subsequent report published by this group (Albro *et al.*, 1983) does not provide any clue to solve the above controversy, but their new findings with alcohol-dual-labeled DEHP at least allowed the conclusion that the intact 2-ethylhexanol side chain cannot simply be attached to DNA. They showed, in addition, that urea isolated from the urine was

$^{14}C$ -labeled, suggesting that the DNA could have been radiolabeled via biosynthesis.

### *Influence of the Pretreatment*

The specific activity of DNA, most if not all due to biosynthetic incorporation of radiolabeled breakdown products, was not affected by DEHP pretreatment of the rats or by DEHA pretreatment of the mice for 4 weeks with 1% in the diet. The liver weight, on the other hand, was increased after pretreatment. This finding means that DNA synthesis per unit liver weight was no longer stimulated after 4 weeks but seemed to be in stationary phase no different from controls.

The fraction of the radioactivity dose exhaled in the form of  $^{14}CO_2$  was larger after pretreatment by factors of 1.4 (DEHP I), 2.1 (DEHP II), and 1.5 (DEHA V). The liver was enlarged by factors of 1.5 (DEHP I) and 1.4 (DEHP II) in the rat and 1.3 (DEHA V) in the mouse. The metabolic activity per unit liver weight therefore did not increase at all or only to a very small extent. DEHP therefore seems not to be an effective inducer of its own metabolism. This finding confirms indirectly other data which show that peroxisomes can be induced without a marked effect on some cytochrome *P*-450-dependent activities (Walseth *et al.*, 1982).

### *The Covalent Binding Index*

The expression of a DNA binding normalized by the dose in the units of the covalent binding index, CBI, was introduced in 1977 (Lutz and Schlatter, 1977a) for a comparison of the DNA-binding activities of various chemicals tested in a number of laboratories under widely different conditions. The values measured so far span about five orders of magnitude, ranging from more than  $10^4$  [aflatoxin  $B_1$  (Lutz *et al.*, 1980)] down to about one [e.g. benzene (Lutz and Schlatter, 1977b)]. CBIs have been shown to reflect very roughly

the genotoxic potency of a chemical. CBIs of the order of  $10^3$  to  $10^4$  are found with potent carcinogens, of around  $10^2$  for moderate carcinogens, and of 1 to 10 for weak carcinogens with a genotoxic mode of action. A plot of the CBI in the target organ as a function of the respective tumorigenic potency in TD50 units derived from long-term bioassays on carcinogenicity has been set up with 13 chemicals for which both values were published (Lutz, 1982a). The surprisingly high correlation coefficient of 0.74 calculated from a linear regression analysis therefore seems to allow placement of a test compound into a category according to the importance of DNA binding as one factor contributing to the overall tumorigenicity.

Our results allow the conclusion that the liver DNA-binding ability of DEHP in rats and of DEHA in mice must be well below a value of 0.05, expressed in CBI units. This is almost one million times below the genotoxicity of aflatoxin B<sub>1</sub>, where daily doses of as little as  $10^{-5}$  mmol/kg resulted in detectable tumor formation. If the much lower maximum possible DNA binding by DEHP and DEHA should become responsible for a positive result in a rodent bioassay on carcinogenicity, impossibly high doses or an unrealistically large number of animals would be required if the correlation of carcinogenic potency to DNA binding is extrapolated to a CBI value of 0.05 (Lutz, 1982a).

The fact that a tumorigenicity of DEHP and DEHA has clearly been demonstrated, however, is an indication that activities other than DNA binding of the test compound must be responsible. The observation that induction of peroxisomes is associated with increased hepatic tumor formation (Reddy *et al.*, 1980) and the finding of an excessive accumulation of autofluorescent lipofuscin in the liver during hepatocarcinogenesis by peroxisome proliferators (Reddy *et al.*, 1982) led these authors to the hypothesis that persistent proliferation of peroxisomes and increase in peroxisomal  $\beta$ -oxidation systems could serve as an endog-

enous initiator of the neoplastic transformation of liver cells by increasing the intracellular production of DNA-damaging H<sub>2</sub>O<sub>2</sub> and other reactive oxygen intermediates.

A DNA-binding assay *in vivo* finds an important application where a long-term assay on carcinogenicity was clearly positive, but where additional information, such as mutagenicity data, renders a genotoxic mode of action via DNA binding unlikely (Lutz, 1982b). These requirements are met with DEHP and DEHA. Here, it was important to verify the lack of DNA binding of the compound also in a mammalian organism. Negative results as shown above suggest that the tumorigenicity might have been dependent on the onset of some type of biological response which should also have been observable in the bioassay. With DEHP and DEHA, this response could well be the proliferation of peroxisomes. For an assessment of the risk for man from exposure to this type of carcinogen, it will therefore be important to establish dose-effect relationships and to further substantiate the importance of species differences.

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