The relevance of covalent binding to mouse liver DNA to the carcinogenic action of hexachlorocyclohexane isomers

Peter Sagelsdorff, Werner K. Lutz1 and Christian Schlatter
Institute of Toxicology, ETH and University of Zürich, CH-8603 Schwerzenbach, Switzerland.

(Received on 20 April 1983; accepted on 15 July 1983)

Abstract

[1H]Hexachlorocyclohexane (HCH) was synthesized by chlorination of [1H]benzene prepared by catalytic tritiation of benzene with tritiated water. The isomers of HCH were separated by adsorption chromatography on silica gel. In order to determine the covalent binding to DNA, [1H]HCH was administered to male mice by oral gavage, and liver DNA was isolated via chromatography. The specific radioactivity of the DNA was normalized by the dose administered and expressed in the molar units of the Covalent binding index, CBI = DNA damage/dose (μmol bound HCH/mol DNA nucleotide)/(μmol HCH administered/kg body weight). CBI values of -0.2 were found 10 h after the administration of alpha- and gamma-HCH. Enzymatic digestion of the DNA to the nucleosides and the nuclear acids revealed that -40% of the radioactivity is co-migrated with the natural nucleosides. Aliquots volumes known to contain the more lipophilic carcinogen-nucleoside adducts, -10% of the radioactivity could be detected. The remaining 50% of the radioactivity eluted with the front, representing a mixture of oligonucleotide-HCH adducts and/or hydrophilic degradation products which were strongly but not covalently associated with intact DNA. Therefore, a true CBI of 0.02 - 0.01 must be expected both for alpha- and gamma-HCH. This CBI is by a factor of 103 - 104 below the value found with the strongest DNA-binding carcinogens like aflatoxin B1 or dimethylhydrazine and is unlikely to be decisive for the liver tumor induction in mice because of the following additional findings: (i) Both isomers gave rise to similar levels of DNA damage although the alpha-isomer is a much more potent tumor inducer. This similarity was not only at the time of maximum binding but up to 10 days after oral administration; (ii) three mouse strains with apparently different susceptibility to tumor induction by gamma-HCH could not be distinguished with respect to DNA binding; (iii) the level of DNA binding of alpha-HCH (CBI = 0.02 - 0.1) is more than three orders of magnitude lower than would be expected if the mechanism of tumor induction was by genotoxicity mediated by DNA-binding. For a preliminary investigation on a potential stimulatory effect on liver DNA replication and cell division, [3H]thymidine was administered i.p. 3.5 h before sacrifice of the [1H]HCH-treated mice. The alpha-isomer was found to be more potent than the gamma-isomer in this respect. Taken together, our data allow the conclusion that the non-mutational processes must be more important for the carcinogenicity of HCH.

Introduction

Hexachlorocyclohexane (HCH)* comprises of a group of isomers of which the gamma-isomer, later called lindane, has very useful pesticidal activity (1). HCH have become of great public concern because the lindane batches used in the late forties contained appreciable concentrations of alpha- and beta-isomer. The alpha-isomer was found to induce liver tumors in rats and mice (2), the beta-isomer was found to have very low biodegradability and to be deposited in animal fat. Although the lindane batches used since the fifties were at least 99% pure gamma-isomer, a new discussion arose from controversial findings of a liver tumor-inducing potential of lindane itself.

Chemically-induced tumors are now thought to be the result of a DNA damage succeeded by appropriate promotion (3). Most chemicals exert their activity by covalent interaction of a reactive metabolite with DNA in the target organ and are therefore called genotoxic. The metabolism of HCH involves the formation of olefins (1) and a subsequent epoxidation could result in the generation of an electrophilic species.

Another group of tumor-enhancing agents, viz co-carcinogens and promoters, do not themselves react with DNA but apparently modulate one or several out of a variety of biochemical and biological steps related to the process of tumor formation. Such activities are also discussed for HCH. For instance, alpha-HCH was found to enhance the proliferation of putative preneoplastic cells in rat liver (4), and all HCH isomers are known to be inducers of drug-metabolizing enzymes (1), the alpha-isomer being more potent than lindane.

It was the aim of this study to provide more information about the mechanism of tumor induction by HCH. For this reason it was examined whether the isomers of HCH can be metabolized in vivo to reactive metabolites able to react and bind to liver DNA or whether the hepatocarcinogenicity is rather due to non-genotoxic effects. It seemed especially worthwhile to investigate whether the clear difference between the alpha- and the gamma-isomer with respect to biological effects and tumor induction (2) was reflected in their ability to bind to DNA, and whether the apparent difference in susceptibility of different strains of mice to the carcinogenicity of gamma-HCH (5 - 7) can be based upon different levels of DNA binding.

Materials and Methods

Chemicals and apparatus

Reagents without specified distributor were of the highest purity available from Merck, Darmstadt, FRG. Hydroxylapatite (HA) DNA-Grade, Bio-Gel HTP was purchased from Bio-Rad, Richmond, CA, sodium dodecyl sulfate (SDS) from Sigma, St. Louis, MO, Nonidet P 40 (NP 40) and copper oxide (wire form) from BDH Chemicals Ltd., Poole, UK. Carrierfree [3H] and [14C]thymidine with a specific activity of 61 mCi/μmol were purchased from the Radiochemical Centre, Amersham, UK. Desoxyribonuclease I (E.C. 3.1.4.5.) from bovine pancreas, phosphodiesterase I (E.C. 3.1.4.1.) from Crotalus atrox venom and alkaline phosphatase III (E.C. 3.1.3.1.) from Escherichia

*Abbreviations: HCH, hexachlorocyclohexane; CBI, Covalent binding index; PBI, Protein binding index; H, Incorporation index; CIP, chloroform/isoamyl alcohol/phenol (24 + 1 + 25 vol.); SDS, sodium dodecyl sulfate; HA, hydroxylapatite.


1To whom correspondence should be addressed.
isolated from Sigma, St. Louis, MO. Dialysis tubing (Visking type 20/32, mol. wt. exclusion at 12 000−14 000 daltons; diameter 17 mm) was from Union Carbide, Chicago, IL. The u.v. lamp was a Minilight UVSL-58 50W from Ultra Violet Products Inc., San Gabriel, CA.

Radioactivity measurements were carried out in 10 ml Insta-Gel (Packard Instrument, Downers Grove, IL) in a liquid scintillation counter, Packard Tri Carb 460 CD equipped with and calibrated for the automatic analysis of [3H]PCl2 double-labelled samples.

The isometric and radioactive purities of the isomers of HCH were determined on a semipreparative gas chromatograph, Carlo Erba, Fratocap Lina 2200 (Carlo Erba, Rodana, Milano, Italy). The mass spectrum of HCH was recorded at the Institute of Organic Chemistry, ETH Zürich, Switzerland. The h.p.l.c. analysis of the nucleosides was performed on a semipreparative column (250 mm x 8 mm I.D.) equipped with two h.p.l.c. pumps, Kontron, LC Pump 410 (Kontron, Zürich, Switzerland), controlled by a Kontron Programmator 200, for generating a linear gradient of two eluants.

**Synthesis of [3H]HCH**

[3H]Benzene was prepared by analytic exchange titration of benzene on a high-vacuum line. [3H]Clorobenzene resulting from combating 8.8 Ci carrier-free [3H]Cl, at 60°C over 30 g copper oxide (wire form) was trapped under high vacuum in 250 µl trifluoroacetic acid cooled with liquid nitrogen. This mixture was lyophilized and was obtained from oxidation of 33 mmol silver chloride with 12 g potassium dichromate in 100 ml concentrated sulfuric acid and trapped by cooling with liquid nitrogen. The [3H]benzene and chlorine were hypolized into a 25 ml quartz round-bottom flask pre filled with 5 ml carbon tetrachloride. The mixture was kept at −20°C to −30°C for 2 h under irradiation at 254 nm. Solvent and unresolved benzene was distilled off and the residue containing −50% of the radioactivity was extracted with petroleum ether (30−45°C boiling point) to bring the alpha-, gamma-, delta- and epsilon isomers into solution. The specific radioactivity of HCH was calculated from results derived in preliminary synthesis with a trace amount of radioactivity and was found to be −1 Ci/mmol. The products were identified as HCH by radioactivity measuremmts in vacuo.

Radioactivity was extracted with petroleum ether. A 60% solution of the radioactivity in the aqueous solution was decanted. The remaining slurry was poured into 25 x 120 mm glass columns and the MUP was let run off. The aqueous nucleic acid solution was loaded on the column and the elution was monitored at 260 nm. Proteins were washed from the column with filtered MUP at a flow rate of 1−2 ml/min by gravity until the transmission had returned to background value. To avoid a mixing of the eluants the column was let run dry before purging the area of the column with two bed volumes 14 mM sodium phosphate buffer, pH 6.8. DNA was eluted with 0.48 M sodium phosphate buffer, pH 6.8, and −20 ml of the DNA solution were collected. From here on, extreme caution is required not to use glassware, equipment or facilities which are also used for procedures involving high radioactivity levels. The sample was dried under vacuum at 4°C against 10 liter 0.2 M NaCl overnight. DNA was precipitated by adding 2 volumes ethanol and keeping at −20°C for at least 12 h. The DNA was centrifuged for 30 min at 1000 g, the supernatant was discarded, 1 litre ethyl ether was added and the DNA was dried in vacuo for 2−3 h. The highly purified DNA was dissolved in 10 mM MgCl2, 10 mM Tris/HCl, pH 7.0. The amount of DNA was determined on the basis of an absorbance of 20 at 260 nm for a solution of 1 mg DNA/ml. The yield of DNA was −1 mg/liver limited by the use of suboptimal amounts of hydroxylapatite. The contamination of the DNA by protein was <0.2% as derived from liver-DNA isolations from animals treated with L-[^3H]methionine or with L-[3H]histidine.

In a control experiment DNA was isolated from the pooled livers of two mice treated with [3H]HCH and [3H]Chlorobenzene. After each purification step DNA was precipitated from an aliquot of the aqueous solution by adding 2 volumes ethanol at −20°C. After centrifugation for 20 min at 1000 g, DNA was dried in vacuo and dissolved in denitrogen water. The amount of protein contaminating the crude DNA was measured by the method of Lowry and Stein (12), the amount of DNA was calculated from the specific [3H]Activity. The specific [3H]Activity = [3H]Activity (protein)/amount of DNA. Isolation of chromatin protein.

1 ml of the first CIP extract from a DNA isolation of 10 g liver was shaken with −5 ml 1% SDS in 14 mM sodium phosphate buffer, pH 6.8. Proteins were precipitated by adding 2 volumes ethanol, washed 5 times by redissolving in 2 ml 2% SDS and acetone precipitation. The final protein sample in 1% SDS was diluted with water to 0.1% SDS, was precipitated by the addition of 2 volumes acetone and was stored at −20°C overnight. After centrifugation at 300 g, the supernatant was discarded and the residue was freed from acetone in vacuo for −15 min. Protein was dissolved in 2 ml 1% SDS in 14 mM sodium phosphate buffer, pH 6.8, overnight and the solution was diluted with water to a final concentration of 1.4 mM sodium phosphate buffer. The amount of protein was determined with the Folin reagent. 1−4 ml containing −0.5 mg protein/ml were used for the liquid scintillation counting.

**Isolation of HCH metabolites**

The supernatant of the first acetone precipitation of chromatin protein from the CIP phase was dried in vacuo. About 75% of the radioactivity in the CIP phase could be dissolved in 10 mM MgCl2, 10 mM Tris/HCl, pH 7.0 and was loaded on a Lichrosorb RP18 column also used for the analysis of nucleotides by h.p.l.c. (see below).

Water-soluble metabolites were obtained from the aqueous solution after the first CIP extraction of the chromatin homogenate. DNA was precipitated by the addition of 2 volumes ethanol and the supernatant was dried in vacuo. After dissolving the residue in 10 mM MgCl2, 10 mM Tris/HCl, pH 7.0 the sample was analysed by h.p.l.c.

**h.p.l.c. analysis of the nucleotides**

DNA (1−2 mg/ml) in 10 mM MgCl2, 10 mM Tris/HCl, pH 7.0 was digested enzymatically by the methods described by King et al. (13). The resulting deoxyribonucleotides were separated by h.p.l.c. on a Lichrosorb RP18 column.
column (250 mm x 8 mm) with a distilled water/methanol gradient of 0–10% methanol in 5 min, 10% methanol for 5 min and 10–100% methanol in 45 min at a flow rate of 3.5 ml/min. The optical density of the eluate was recorded at 254 nm. Fractions of 2 min were collected and the scan-

culation counting was performed after the addition of 10 ml Insta-Gel. To

avoid phase separation between the scintillation cocktail and the eluate, the

fractions 11–14 were diluted with 1 ml methanol. The retention times of the

natural deoxynucleosides deoxyadenosine, thymidine and deoxyguanosine were 9 min, 11.5 min, 13 min and 18 min, respectively. Recovery of radioactivity eluted, as a fraction of the injected range between 90 and 110%, both for [PH] and [PC].

Calculations and statistics

Determination of CBI. The radioactivity in the DNA after treatment of mice with [PH]HCH was expressed after normalization to the dose admin-
mistered:

\[
\text{CBI} = \frac{\text{d.p.m./mg DNA}}{\text{d.p.m./kg body weight}}
\]

This value was converted to the molar units,

\[
\text{CBI} = \frac{\mu \text{mol chemical bound/mol DNA nucleotide}}{\text{d.p.m./kg body weight}}
\]

according to CBI = CBI’ x 309 x 10^6 on the basis of an average mol. wt. of 309 g/mol DNA nucleotides (14).

Determination of Protein binding indices, PBI. The radioactivity in the chromatin protein after treatment of mice with [PH]HCH was expressed after normalization to the dose administered:

\[
\text{PBI} = \frac{\text{d.p.m./mg chromatin protein}}{\text{d.p.m./kg body weight}}
\]

This value was converted to the molar units,

\[
\text{PBI} = \frac{\mu \text{mol chemical bound/mol amino acid}}{\text{d.p.m./kg body weight}}
\]

according to PBI = PBI’ x 110 x 10^6 on the basis of a mol. wt. of 110 g/mol for an average amino acid (15).

Determination of Incorporation indices, II. The radioactivity in the DNA after treatment of mice with [1H]thymidine was converted to the molar units,

\[
\text{II} = \frac{\text{d.p.m./mg DNA}}{\text{d.p.m./kg body weight}}
\]

This value was converted to the molar units,

\[
\text{II} = \frac{\mu \text{mol thymidine incorporated/mol DNA nucleotide}}{\text{d.p.m./kg body weight}}
\]

according to II = II’ x 309 x 10^6 on the basis of an average mol. wt. of 309 g/mol DNA nucleotides.

Calculation of standard deviation. The total variability (statistical count-

error and fluctuations due to vial, scintillation cocktail, counter, external radiation and composition of the sample) for the counting of DNA sample containing little radioactivity was assumed to be equal to the variability of DNA samples isolated from untreated animals held together with the treated ones. On the basis of 33 background values compiled for 12 months, a respective standard deviation of 1.89 c.p.m. (± 1 S.D.) was calculated. The stand-

dard deviation for a net radioactivity in a vial therefore was taken as: 1 S.D. = √(1.89^2 + 1.33^2) = 1.92 c.p.m.

Limit of detection for radioactivity in nucleoside analysis. The total variability for each fraction of a h.p.l.c. analysis was calculated on a level of 1 standard deviation (± S.D.) from 5 nucleoside analyses of control DNA digests. The mean background value for each fraction was calculated with an accuracy of 1 S.D./√5. The maximum possible difference between sample and background radioactivity was determined on an interval of ± 2 S.D.

Results

Comparison of isomers

Table I compiles the radioactivities in mouse liver DNA isolated 10 h after oral administration of a high radioactivity dose of gamma- and alpha-[PH]HCH. Under the assumption that the radioactivity is due to DNA-bound HCH molecules, the radioactivity can be expressed in the units of the CBI (14) after normalization to the dose administered. The last line of the Table I reveals the extremely low level of apparent DNA binding (CBI around 0.2) and shows that the alpha-isomer did not give rise to a higher CBI than the gamma-isomer although the former is a markedly more potent carcinogen. This is the first indication to postulate that DNA binding cannot be the decisive activity for the tumor-inducing potential of the HCH's. This hypothesis is also supported by the finding that the delta-isomer which has never been found to induce tumors (16) also revealed a CBI of 0.16 (Table II). The beta-isomer did not give rise to detectable DNA radioactivity (Table II).

In order to check whether a difference between the gamma- and the alpha-isomer might be found at later times after the administration, the time dependence for the DNA binding was investigated. Figure 1 shows that the liver-DNA radioactivity reached the same plateau level of about CBI = 0.5 after 10 days. The time-dependent increase was faster in the first three days with the gamma-isomer, in accordance with the somewhat faster metabolism (17). It was also checked whether the absorption from the gastro-intestinal tract of the relatively high doses of chemical was different for the two isomers. This was not the case because it was found that the whole liver contained 2.1% and 2.2% of the radioactivity dose of the gamma- and the alpha-isomer, respectively, one day after the oral administration.

Comparison of mouse strains

An additional hint for whether DNA binding could be the main mode of tumorigenic action of HCH should be obtained from studies with strains of mice that are of apparently different susceptibility to liver tumor induction by the gamma-isomer. The results given in Table III show that the three strains used all gave rise to similar CBI values although NMRI mice (CBI = 0.28) were found to be less susceptible to the tumorigenic action of gamma-HCH than B6C3F1 mice
formed to investigate some of the above-mentioned contributions.  

Contamination by protein-bound HCH. The data given in Table IV show that chromatin protein was also radio-labelled, 10 h after [3H]HCH administration. The specific activity was 42- to 68-fold and 33- to 44-fold higher in protein than with DNA for the gamma-, and the alpha-isomer, respectively. Protein contamination of DNA was shown to be lower than 0.2% as determined with radiolabelling of chromatin protein in vivo with [3H]lysine or [35S]methionine. Protein contaminations cannot therefore contribute substantially to the radioactivity measured on the DNA.

Contamination of DNA by non-covalently bound HCH metabolites. The control experiment where DNA was precipitated at different steps of the purification procedure showed that neither ether extraction nor dialysis resulted in a reduction of the radioactivity of DNA. To determine whether there was non-covalent binding of metabolites on DNA, DNA was isolated from the liver of a gamma-[3H]HCH-treated mouse (CBI = 0.17) or CF1 mice (CBI = 0.26) (5-7).

Table IV. Specific activity of liver chromatin protein of male NMRI mice, 10 h after oral administration of [3H]HCH.

<table>
<thead>
<tr>
<th>Isomer</th>
<th>gamma-HCH</th>
<th>alpha-HCH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal weight</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[g]; pool of 2 mice</td>
<td>66.2</td>
<td>70.2</td>
</tr>
<tr>
<td>Dose [mg/kg]</td>
<td>13.0</td>
<td>12.0</td>
</tr>
<tr>
<td>[mCi/kg]</td>
<td>44.9</td>
<td>41.5</td>
</tr>
<tr>
<td>Protein Sp. act. [d.p.m./mg]</td>
<td>6140</td>
<td>5170</td>
</tr>
<tr>
<td>[PBI units]</td>
<td>6.8</td>
<td>6.2</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>5.6 ± 0.9</td>
<td>2.3 ± 0.1</td>
</tr>
</tbody>
</table>

The control protocol was repeated to investigate some of the above-mentioned contributions.  

Contamination by protein-bound HCH. The data given in Table IV show that chromatin protein was also radio-labelled, 10 h after [3H]HCH administration. The specific activity was 42- to 68-fold and 33- to 44-fold higher in protein than with DNA for the gamma-, and the alpha-isomer, respectively. Protein contamination of DNA was shown to be lower than 0.2% as determined with radiolabelling of chromatin protein in vivo with [3H]lysine or [35S]methionine. Protein contaminations cannot therefore contribute substantially to the radioactivity measured on the DNA.

Contamination of DNA by non-covalently bound HCH metabolites. The control experiment where DNA was precipitated at different steps of the purification procedure showed that neither ether extraction nor dialysis resulted in a reduction of the radioactivity of DNA. To determine whether there was non-covalent binding of metabolites on DNA, DNA was isolated from the liver of a gamma-[3H]HCH-treated mouse (CBI = 0.17) or CF1 mice (CBI = 0.26) (5-7).

Table IV. Specific activity of liver chromatin protein of male NMRI mice, 10 h after oral administration of [3H]HCH.

<table>
<thead>
<tr>
<th>Isomer</th>
<th>gamma-HCH</th>
<th>alpha-HCH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal weight</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[g]; pool of 2 mice</td>
<td>66.2</td>
<td>70.2</td>
</tr>
<tr>
<td>Dose [mg/kg]</td>
<td>13.0</td>
<td>12.0</td>
</tr>
<tr>
<td>[mCi/kg]</td>
<td>44.9</td>
<td>41.5</td>
</tr>
<tr>
<td>Protein Sp. act. [d.p.m./mg]</td>
<td>6140</td>
<td>5170</td>
</tr>
<tr>
<td>[PBI units]</td>
<td>6.8</td>
<td>6.2</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>5.6 ± 0.9</td>
<td>2.3 ± 0.1</td>
</tr>
</tbody>
</table>

The control protocol was repeated to investigate some of the above-mentioned contributions.  

Contamination by protein-bound HCH. The data given in Table IV show that chromatin protein was also radio-labelled, 10 h after [3H]HCH administration. The specific activity was 42- to 68-fold and 33- to 44-fold higher in protein than with DNA for the gamma-, and the alpha-isomer, respectively. Protein contamination of DNA was shown to be lower than 0.2% as determined with radiolabelling of chromatin protein in vivo with [3H]lysine or [35S]methionine. Protein contaminations cannot therefore contribute substantially to the radioactivity measured on the DNA.
were analysed on the same h.p.l.c. system as used for the nucleosides. Figure 3 shows that the hydrophilic metabolites eluted in the first three fractions. Since this is the region which also contained radioactivity in the h.p.l.c. analysis of the DNA nucleoside it cannot be excluded that such metabolites were closely but non-covalently associated with DNA. The association must have been so strong that the dialysis did not remove them and only upon enzymatic degradation of the DNA were they released.

Lipophilic metabolites eluted at a retention time of ~50 min. No radioactivity could be detected at retention times between 24 min and 48 min. These data indicate that the radioactivity peaks eluting after ~35 min represent HCH-deoxynucleoside-adducts and not metabolites set free during the hydrolysis of the DNA.

True covalent DNA binding

The control experiments described above have revealed that biosynthetic incorporation of radiolabel into DNA has taken place and that hydrophilic metabolites might have been strongly but non-covalently associated with DNA. For a calculation of a true CBI, these contributions have to be deducted from the values given in Tables I–III. A reduction by a factor of ~2 or 10 results, for the case where all early-eluting radioactivity is regarded as oligonucleotide-HCH adducts or for the case where only the nucleoside-HCH adduct peak is considered. On a most conservative approach, therefore, a CBI of <0.1 results.

Protein binding

In our experiments on DNA binding we also determined the level of non-extractable radioactivity in chromatin protein. This was done in a first place in order to determine whether contamination of DNA with protein of high specific radioactivity might simulate DNA binding. The protein-binding values are also low on an absolute level upon comparison with standard carcinogens (15), and, there was again no difference in protein binding between the two isomers (Table IV) or between the different strains of mice (Table V).

Rate of DNA synthesis

The animals received, 3.5 h before sacrifice, an i.p. injection of [14C]thymidine. The level of [14C]radioactivity on the DNA was then used as an index for DNA synthesis. The results summarized in Table VI show a tendency for the more potent alpha-isomer to induce a higher rate of DNA synthesis. It should be pointed out that these data were obtained from the identical animals used for the determination of DNA binding by [3H]HCH (Table I), where the alpha-isomer was even slightly less effective than the gamma-isomer.
cannot be an important contribution to the proposed non-envisaged production of to an assay within the present experimental set-up. Since the experimental set-up was not ideal for the determination of binding to protein and the concomitant cytotoxicity might be (19,20). Although the experimental set-up was not ideal for the determination of (18). Since alpha-HCH has to be classified as a moderate carcinogens and of (22). As suggested by Radman and Kinsella (23) there may not be a clear separation of these two groups of activities and it must be expected that there are carcinogens acting on more than only one level. With HCH, we are faced with a situation where a minute DNA binding was shown for the alpha- and the gamma-isomer. Much additional information was given, however, to show that this activity cannot be responsible alone for the tumors induced and some hypotheses were presented and in part supported by experimental data. For a risk evaluation in man, a mutagenic risk by DNA binding after exposure to mg amounts of lindane seems negligible. Although species differences with respect to the formation of reactive metabolites cannot be excluded we do have evidence that binding of gamma-HCH to liver DNA in the rat is similar to the data obtained in mice so that there are at least no indications for large species differences with respect to metabolism. For the more important events not related to DNA binding, an extrapolation of animal data to man must be based upon the elucidation of the specific mechanism of tumor induction by HCH in the animal, and a test of whether similar activities are found in man at dose levels that are by orders of magnitude lower than those used in the long-term bioassays.

Acknowledgement
This study was supported by the 'Centre International d'Etudes du Lindane', C.E.E.L., Brussels, Belgium.

References

Discussion
The previous section has provided good qualitative evidence for a DNA – HCH adduct. A comparison among strains and isomers made it highly unlikely, however, that this type of genotoxic activity is the decisive mode of tumorigenic action. In addition, a quantitative analysis of the level of DNA binding favours a non-genotoxic mode of tumorigenic action. A quantitative correlation of CBI versus carcinogenic potency expressed in TD50 units (i.e., the daily dose estimated to induce a tumor in 50% of the animals treated for life), has shown that CBI of the order of 10^10 are found with strong genotoxic carcinogens, of 100 for moderate carcinogens and of 1–10 for weak carcinogens (18). Since alpha-HCH has to be classified as a moderate tumor-inducing agent with an approximate TD50 value of 1 mmol/kg/day for mouse liver, a CBI for liver DNA of ~10^10 would be required if its mode of action was by DNA binding. The measured value of <0.1 is one thousand times lower.

Among the many possible mechanisms of tumorigenic activity not related to DNA binding two aspects were amenable to an assay within the present experimental set-up. Firstly, binding to protein and the concomitant cytotoxicity might be envisaged. Our results showed, however, that protein binding cannot be an important contribution to the proposed non-mutagenic mode of tumor induction.

Another possibility for a non-mutagenic mode of action, the stimulation of cell division, was also tested simultaneously with the determination of DNA binding. These results gave some indication for a higher activity of the alpha-isomer although the experimental set-up was not ideal for the determination of this activity. It has been shown in rats that the induction of DNA synthesis after administration of various tumor promoters is largest after a time period of ~20 h (19,20). In our experiments, we used mice and the interval was only 6.5 h. Therefore, the borderline effect observed with the alpha-isomer might have become more pronounced if a time dependence had been investigated. Additional evidence along these lines is available from two-stage long-term carcinogenicity studies with rats where it has been shown that alpha-HCH accelerated the manifestation of malignant liver tumors after initiation of the carcinogenic process by a single dose of diethylnitrosamine (21). Chemical carcinogens are normally divided into two large classes of mutagenic and non-mutagenic carcinogens (22). As suggested by Radman and Kinsella (23) there may not be a clear separation of these two groups of activities and it must be expected that there are carcinogens acting on more than only one level.

Table V. Specific activity of liver chromatin protein of male NMRI, CFl and B6C3Fl mice, 10 h after oral administration of gamma-[3H]HCH.

<table>
<thead>
<tr>
<th>Strain</th>
<th>NMRI</th>
<th>CFl</th>
<th>B6C3Fl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal weight [g]; pool of 2 mice</td>
<td>87.9</td>
<td>91.7</td>
<td>73.5</td>
</tr>
<tr>
<td>Dose [mg/kg]</td>
<td>8.7</td>
<td>16.7</td>
<td>21.2</td>
</tr>
<tr>
<td>[μCi/kg]</td>
<td>30.0</td>
<td>57.7</td>
<td>73.0</td>
</tr>
<tr>
<td>Protein Sp. act.</td>
<td>3300</td>
<td>7610</td>
<td>970</td>
</tr>
<tr>
<td>[PBI units]</td>
<td>5.5</td>
<td>6.5</td>
<td>5.4</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>6.0 ± 0.5</td>
<td>5.8 ± 0.5</td>
<td>2.9 ± 0.02</td>
</tr>
</tbody>
</table>

Table VI. Incorporation of [3H]thymidine into liver DNA of male NMRI mice, 3.5 h after i.p. injection, and 10 h after oral application of [3H]HCH.

<table>
<thead>
<tr>
<th>Isomer</th>
<th>gamma-HCH</th>
<th>alpha-HCH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal weight [g]; pool of 2 mice</td>
<td>66.2</td>
<td>70.2</td>
</tr>
<tr>
<td>Dose [μCi/kg]</td>
<td>40.0</td>
<td>30.5</td>
</tr>
<tr>
<td>[μCi/kg]</td>
<td>10.0</td>
<td>7.6</td>
</tr>
<tr>
<td>DNA Sp. act.</td>
<td>184</td>
<td>149</td>
</tr>
<tr>
<td>[d.p.m./mg]</td>
<td>2560</td>
<td>2710</td>
</tr>
<tr>
<td>Incorp. Index</td>
<td>2600 ± 60</td>
<td>3500 ± 400</td>
</tr>
</tbody>
</table>

Table of incorporation of [3H]thymidine into liver DNA of male NMRI mice, 3.5 h after i.p. injection, and 10 h after oral application of [3H]HCH.