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DIFFERENTIATION OF THE MECHANISMS OF ONCOGENICITY OF 1,4-DIOXANE AND 1,3-HEXACHLOROBUTADIENE IN THE RAT

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This report is: ☐ INTERIM ☒ FINAL and mainly: ☒ NEW ☐ REVIEW

DESCRIPTIVE SUMMARY WITH CONCLUSIONS:

1,4-Dioxane (DX) and 1,3-hexachlorobutadiene (HCBD) were studied relative to their probable mechanisms of tumorigenicity in male Sprague-Dawley rats. Upon repeated exposure of rats to tumorigenic dosage levels of DX (1000 mg/kg/day for 11 weeks via drinking water) or HCBD (20 mg/kg/day for 3 weeks orally), histological and biochemical evidence of cellular toxicity was observed in their respective target organs, liver (DX) and kidney (HCBD). These observations correlate well with the chronic bioassays indicating that tumorigenicity was associated only at dose levels which were cytotoxic. This implies that tissue injury (cytotoxicity) may contribute to the carcinogenic process. While no genetic effects were observed in the liver with DX, some genetic damage (DNA repair) and slight alkylation of kidney DNA was observed in rats dosed with 20 mg HCBD/kg. Thus the lack of any significant genetic acting potential for dioxane suggests that dioxane may cause tumors via a nongenetic mechanism, possible through repeated tissue injury and/or the enhancement of the expression of pre-existing oncogenic factors through a phenobarbital-like enzyme induction. While HCBD appears to cause tumors, also primarily via a nongenetic mechanism probably by repeated tissue injury at high dosages, a minor genetic contribution to the carcinogenic effect of HCBD cannot be ruled out at this time.

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DIFFERENTIATION OF THE MECHANISMS OF ONCOGENICITY OF 1,4-DIOXANE AND 1,3-HEXACHLOROBUTADIENE IN THE RAT

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ABSTRACT

1,4-Dioxane (DX) and 1,3-hexachlorobutadiene (HCBD) were studied relative to their probable mechanisms of tumorigenicity in male Sprague-Dawley rats. Upon repeated exposure of rats to tumorigenic dosage levels of DX (1000 mg/kg/day for 11 weeks via drinking water) or HCBD (20 mg/kg/day for 3 weeks orally), histological and biochemical evidence of cellular toxicity was observed in their respective target organs, liver (DX) and kidney (HCBD). These observations correlate well with the chronic bioassays indicating that tumorigenicity was associated only at dose levels which were cytotoxic. This implies that tissue injury (cytotoxicity) may contribute to the carcinogenic process. While no genetic effects were observed in the liver with dioxane, some genetic damage (DNA repair) and slight alkylation of kidney DNA was observed in rats dosed with 20 mg HCBD/kg. Thus the lack of any significant genetic acting potential for dioxane suggests that dioxane may cause tumors via a nongenetic mechanism, possibly through repeated tissue injury and/or the enhancement of the expression of pre-existing oncogenic factors through a phenobarbital-like enzyme induction. While HCBD appears to cause tumors, also primarily via a nongenetic mechanism probably by repeated tissue injury at high dosages, a minor genetic contribution to the carcinogenic effect of HCBD cannot be ruled out at this time.

INTRODUCTION

1,4-Dioxane (DX) and 1,3-hexachlorobutadiene (HCBD) have been reported to be tumorigenic upon chronic ingestion by rats. DX has been reported to cause hepatocellular carcinomas in rats receiving 0.75-1.8% DX in drinking water for 13 months (Argus et al., 1973; 1975; Hoch-Ligeti et al., 1970) or 1015 mg DX/kg/day in drinking water (Kociba et al., 1974). In the later study dose related hepatic tissue damage and regenerative changes were also observed at carcinogenic and lower dosages (Kociba, et al., 1974). A 2-year rat inhalation study of DX, however, was without pathological or carcinogenic results (Torkelson et al., 1974).

HCBD has reportedly caused an excess of renal tubular adenocarcinomas when fed to rats at a level of 20 mg HCBD/kg/day for 2 years (Kociba et al., 1977). As with DX, nontumor related renal histopathology was evident at this, and at a nontumorigenic dose level (2 mg/kg/day). A positive response of HCBD in the bacterium Salmonella typhimurium TA100 in vitro mutagenesis assay has also been reported (Simmon, 1977). No adverse cytogenetic effects have been observed, however, in bone marrow cells of rats receiving up to 20 mg HCBD/kg/day in their diet for 90 days (Mailhes et al., 1974).

As data regarding the macromolecular interactions of DX and HCBD were lacking, the probable mechanisms of tumorigenicity of these compounds was unknown. Thus, the significance of the high dose chronic rat bioassay results in terms of human risk assessment was difficult to determine. Earlier work with vinylidene chloride (Reitz et al., 1980) and perchloroethylene (Schumann et al., 1980) have indicated that some compounds may cause tumors without directly interacting with genetic material (i.e. via a nongenetic mechanism), for example by repeated tissue damage. Thus the following study was undertaken to determine if DX and HCBD caused tumors in rats primarily via a genetic or nongenetic mechanism. The known genotoxic agent dimethylnitrosamine (DMN) served as a positive control for comparison purposes.

MATERIALS AND METHODS

Chemicals

1,4-Dioxane-UL-^{14}C (specific activity (S.A.) = 5.8 mCi/mmole) and 1,3-hexachlorobutadiene-UL-^{14}C (S.A. = 12.0 mCi/mmole) were obtained from Midwest Research Inst. (Kansas City, MO). Dimethylnitrosamine-Me-^{14}C (S.A. = 12 mCi/mmole) was obtained from Amersham Corp. (Arlington Heights, IL). Thymidine [6-^{3}H] (S.A. = 24.7 Ci/mmole) was purchased from New England Nuclear (Boston, MA). All labelled compounds were checked for radiochemical purity upon receipt and found to be >98% pure. Unlabelled DX was obtained from Baker Chemical Co. (Phillipsburg, NJ), HCBD from Dow Chemical Company (Midland, MI), and DMN from Aldrich Chemical Co. (Milwaukee, WI), and were >99% pure. Hydroxyurea, all enzymes and all nucleosides were obtained from Sigma Chemical Co. (St. Louis, MO).

Animals

Male Sprague-Dawley rats (Spartan Research Lab., Haslett, MI) weighing 180-260g were used in all experiments except in the DX-^{14}C binding experiment where 350g rats were used. Rats were housed in wire cages in environmentally controlled animal holding rooms and supplied Purina Rat Chow (Ralston Purina Co., St. Louis, MO) and water ad libitum. Animals were identified individually by ear tags and were acclimated for $\geq$10 days prior to use.

Statistics

Animals were computer randomized into treatment groups in all experiments and the results compared by Dunnett's t-test (Steel and Torrie, 1960) with the level of significance set at $p < 0.05$.

Acute and repeated dosing of rats with DX or HCBD; DNA synthesis determination.

Rats (4-6/treatment group) were lightly anesthetized with methoxyfluorane and shorn of hair between the shoulder blades. After swabbing with 95% ethanol, a small incision was made and a pouch was constructed anterior to the incision. A model 2001 Alzet capsule (Alza Corp., Palo Alto, CA) loaded with 200 µl of a thymidine [6^{3}H] solution (100 µCi, 0.98 µg in distilled water) was placed inside the pouch. The incision was closed with surgical clips and the animal was allowed to recover $\geq$ 6 hours prior to dosing. For acute studies, DX (0, 10, 100 or 1000 mg/kg) or HCBD (0, 0.2 or 20 mg/kg) in saline and corn oil (USP grade), respectively, were administered by gavage at a volume of 1 ml/kg body wt. Animals were

sacrificed 7 days after dosing by cervical dislocation, and their livers (DX experiments) or kidneys (HCBD experiments) were excised, weighed, sampled for histology (see below) and finally frozen on dry ice. Tissues were stored at -80°C until analyzed. DNA was isolated after the method described by Reitz et al. (1980) and quantitated by the diphenylamine method of Giles and Myers (1965). ^{3}H content of isolated DNA representative of thymidine [6-^{3}H] incorporation was assayed by liquid scintillation spectroscopy using 10 ml ACS scintillation cocktail (Amersham Corp., Arlington Heights, IL).

Animals used in repeated dosing experiments received an average of 0, 10 or 1000 mg DX/kg/day for 11 weeks (7 days/wk) via drinking water, 0, 0.2 or 20 mg HCBD/kg/day for 3 weeks (7 days/wk) by gavage, or 3 mg DMN/kg/day for 3 weeks (5 days/wk) by gavage. Rats were implanted 7 days prior to termination of the experiment with thymidine [6-^{3}H]-loaded Alzet osmotic pumps as previously described. With the exception of dosing, all procedures were conducted as described in the acute experiments.

DNA alkylation *in vivo*: DNA isolation and high pressure liquid chromatography analysis.

Rats were dosed with either 1000 mg DX-^{14}C/kg (approximately 570 µCi/rat, S.A. = 0.143 mCi/mmole), 20 mg HCBD-^{14}C/kg (approximately 366 µCi/rat, S.A. = 12.0 mCi/mmole), or 3 mg DMN-^{14}C/kg (approximately 15 µCi/rat, SA = 1.09 mCi/mmole), by gavage and sacrificed by decapitation 4 hr (DX, DMN) or 6-30 hr (HCBD) later. DNA was isolated from the tissues by modifications of the Marmur (1961) and Kirby (1957) methods. Briefly this involved homogenization of the tissue in a Potter-Elvehjem type vessel in 5 vol. 0.15M NaCl-0.1M EDTA Buffer (pH 8.0), deproteination with the chloroform:isoamyl alcohol (96:4)-5M $NaClO_4$ technique of Marmur (1961), precipitation of DNA in an equal volume of 95% ethanol and removal of glycogen and RNA using the methoxy and ethoxy ethanol techniques of Kirby (1957). Samples were then incubated at 37°C in 0.15M NaCl-0.015M citrate buffer (pH 7.0) containing 1 mg/ml α-amylase and RNAase A. After 1 hr, trypsin and chymotrypsin were added to bring the concentration to 1 mg/ml sample and incubation was continued for 2 more hours. Sodium acetate was then added to 4% (W/V) and the DNA was precipitated with an equal volume of ethoxyethanol. Isolated DNA (recovery ≈ 2 mg/g tissue) was found to be free of detectable RNA (orcinol reaction) and glycogen (anthrone reaction). Quantitation of the DNA and the ^{14}C content was accomplished as described above using

small aliquots of the sample. The remaining purified DNA was then digested in a 0.5M TRIS - 0.01M $MgCl_2$ buffer (pH 7.8) using 0.05 mg DNAase I, 1 mg snake venom phosphodiesterase and 1.5 U alkaline phosphatase/mg DNA at 37°C for 16 hr.

Nucleosides were subsequently analyzed by high pressure liquid chromatography (HPLC) on a Partisil M-9 10/50 SCX column (Whatman Inc., Clifton, NJ) with a Waters model 6000A pump (Milford, MA) equipped with a Perkin-Elmer model LC 55B Spectrophotometer (Oak Brook, IL). Nucleosides were eluted using an isocratic flow of 0.3 ml/min (0.1 M $NH_4H_2PO_4$, pH 3.5) and detected at 254 nm wavelength. Fractions (2.5 ml) of the eluate were collected and following addition of 10 ml Aquasol-2 scintillation cocktail (New England Nuclear, Boston, MA) the ^{14}C content of eluate fractions were measured by liquid scintillation spectroscopy.

In vivo DNA repair

In vivo DNA repair was measured in groups of rats receiving either 1000 mg DX/kg, 20 mg HCBD/kg or 20 mg DMN/kg via gavage by a modification of the method outlined by Arfellini and Grilli (1978). Briefly this involved the use of repeated i.p. injections of 500 mg hydroxyurea/kg at 1.5 hour intervals to repress normal DNA synthesis starting 30 min after dosing of the test chemical. Animals also received repeated hourly s.c. injections of 1.0 µg thymidine [6-^3H](100 µCi)/kg starting 1 hr after test chemical dosing for a total of 6 hr. Subsequently, hepatic or renal DNA was isolated and the DNA and ^3H content were quantitated as described above. The results are presented as a repair ratio according to the method of Reitz et al. (1980).

Short term assays

Bacterial mutagenicity assays in Salmonella typhimurium were conducted as described by Ames et al. (1975) using appropriate positive and negative controls. The enzyme activating system (S-9 mix) was a rat liver homogenate obtained from Arochlor 1254-induced animals and purchased from Litton Bionetics (Kensington, MD). HCBD was tested with and without an activating system using Salmonella typhimurium strain TA100 using concentrations of 0 to 5000 µg HCBD (DMSO carrier)/plate. DX was assayed (0 to 103 mg DX in saline/ plate) with and without an activation system using S. typhimurium strains TA 1535, 1537, 1538, 98 and 100 along with appropriate positive and negative control compounds.

The rat primary hepatocyte unscheduled DNA synthesis bioassay (UDS) of DX (10^{-8} to 1M) and HCBD ($3x10^{-8}$ to $3x10^{-2}$M) was done after the method outlined by Williams (1976; 1977).

Histopathology

Tissue samples from exposed animals in acute and repeated dosing experiments were taken from the central region of the animals left kidney (HCBD, DMN experiments) and/or the left lobe of the liver (DX, DMN experiments). Samples were fixed in 10% neutral buffered formalin, sectioned, processed by standard procedures, stained with hematoxylin-eosin dyes and observed microscopically for any evidence of morphologic changes associated with treatment.

RESULTS

As shown in Tables 1 and 2 repeated exposure of rats to tumorigenic dose levels of DX and HCBD were cytotoxic to hepatic and renal tissues, respectively. Conversely, a tumorigenic dose level of DMN (Table 3) failed to produce a measurable increase in cytotoxicity in either tissue relative to controls.

DX did not appear to cause a significant degree of hepatic cytotoxicity in rats when administered as an acute dose (Table 1). No significant changes relative to control animals were noted in organ to body weight ratios, the amount of DNA/g tissue, the rate of DNA synthesis as measured by thymidine [6-^{3}H] incorporation (indicative of cellular regeneration following damage) or the presence of histopathological changes in the livers of rats dosed acutely by gavage with 10, 100 or 1000 mg DX/kg. There was an apparent decrease in weight gain in treated animals presumably due to decreased food consumption consistent with the histological observation that hepatocytes were depleted of glycogen. In contrast, repeated dosing of DX over an 11 week period with an average 1000 mg DX/kg/day induced an increase in liver to body weight ratio and a statistically significant increase (1.5 fold) in hepatic DNA synthesis accompanied by a minimal degree of centrilobular hepatocellular swelling. No changes relative to controls were noted in rats dosed with 10 mg DX/kg/day.

As with DX, doses of HCBD which were tumorigenic to rats upon chronic exposure were cytotoxic in the rat (Table 2). In two acute gavage dosing experiments rats given 0.2 or 20 mg HCBD/kg did not differ from controls in weight gain, kidney to body weight ratio or DNA content/g tissue. In the first trial no changes were observed in renal DNA synthesis or histopathology; but in a second acute trial, a statistically significant increase in DNA synthesis activity was observed in high dose (20 mg/kg) animals. No histopathologic examination was conducted in this group of rats.

Repeated gavage dosing of HCBD for 3 weeks resulted in changes in several measured parameters relative to controls. In animals dosed with 20 mg HCBD/kg/day a statistically significant decrease in weight gain (44%), an increase in kidney to body weight ratio (1.3 fold) and the presence of histopathological changes in renal tissue were observed. A 1.8 fold increase in renal DNA synthesis was also noted in these rats;

however, the large variation between the response of individual animals to the administered HCBD precluded a statistically significant difference when compared to controls. Three of the 5 animals of this group were considerably more sensitive to HCBD as evidenced by their much higher renal DNA synthesis rate and degree of pathologic alterations relative to controls. Histopathological changes were noted in tubular epithelial cells located primarily in the middle and inner cortical region of the kidney. These changes were characterized by the loss of cytoplasm, nuclear pyknosis, increased basophilia, mitotic activity and increased cellular debris within the tubule lumen consistent with the simultaneous degeneration and regeneration of renal tissues. No changes were observed in rats treated with a nontumorigenic dose level of 0.2 mg HCBD/kg/day.

Repeated gavage administration of a tumorigenic dose level of DMN (3 mg/kg/day) for 3 weeks resulted in no significant changes in body weight gains, liver or kidney weight to body weight ratios, DNA content/g tissue, DNA synthesis activities and no observable histopathology in either tissue relative to control rats (Table 3).

As shown in Tables 4 and 5 a small degree of genotoxicity was detected in rats dosed with a tumorigenic dose level of HCBD relative to DMN treated animals. Renal DNA repair increases of 1.27 and 1.54 fold over control animals were observed in the two trials using rats treated with 20 mg HCBD/kg (Table 4). By comparison a 2.87 fold increase in renal DNA repair was observed in rats dosed by gavage with the genotoxin DMN (20 mg/kg). The in vivo DNA repair assay used (Arfellini and Grilli, 1978; Reitz, et al., 1980) allowed for the observation of DNA repair over a 6 hr. period post chemical exposure while a majority of normal DNA synthetic activity was blocked by hydroxyurea. Corrections were then made for treatment related effects and any incomplete supression of normal DNA synthesis.

Consistent with the observation of increases in renal DNA repair as a result of HCBD exposure, isolated renal DNA from rats dosed with 20 mg HCBD-^{14}C/kg were found to be alkylated an average of 0.78 (±0.87) alkylations/10^6 nucleotides (Table 5). Samples collected up to 30 hr post dosing (6, 18.5 and 30 hr) revealed a great deal of variation in the amount of alkylation observed and thus no estimate of the half-life(s) of DNA-HCBD alkylation product(s) could be determined. By comparison renal DNA isolated from rats dosed for 3 weeks with 3 mg

DMN/kg/day (last dose being DMN-^{14}C) was alkylated at a level of 28.3 (±3.14) alkylations/10^6 nucleotides. A renal covalent binding index (CBI;Lutz, 1979) of 10.2 and 698 was calculated for HCBD and DMN respectively (Table 5) (the CBI is a convenient means of comparing the DNA alkylating potency of these and other compounds).

HPLC analysis of isolated nucleosides obtained from the purified renal DNA of rats dosed with 20 mg HCBD-^{14}C/kg revealed a small radioactive peak just preceeding elution of deoxycytosine. The shape and position of this radioactive peak was indicative of a true HCBD-DNA alkylation product. As no ^{14}C peaks were observed in the chromatographic positions where any glycogen, RNA hydrolysis products, or normal nucleosides eluted, the observed ^{14}C peak was not likely due to C-1 incorporation into normal DNA components or trace contamination with RNA or glycogen. However, as the amount of renal DNA sample available for HPLC analysis was limited, the observation of an HCBD-DNA alkylation product could not be confirmed by a second analysis and thus remains unconfirmed.

No mutagenic activity by HCBD was observed in the S. typhimurium TA 100 mutagenesis assay system with or without a metabolic activating system (Table 6). This negative response occurred over a dose range of HCBD (0 to 5 mg/plate) reported to cause up to a 6 fold increase over the background reversion rates in this strain by Simmon (1977). Supportive of the lack of bacterial mutagenicity, no increased UDS response was observed in rat hepatocyte UDS bioassays of HCBD.

Unlike HCBD, DX was not observed to cause an increased rate of hepatic DNA repair in rats treated with a tumorigenic dose level of 1000 mg DX/kg (Table 4). Likewise, no alkylation of hepatic DNA was observed in rats receiving 1000 mg DX-^{14}C/kg with an alkylation detection limit of 1 alkylation/10^6 nucleotides. In contrast a 3.72 fold increase in hepatic DNA repair synthesis was evidenced in rats dosed with 20 mg DMN/kg (Table 4) and an average of 167 (±20.2) alkylation/10^6 nucleotides were detected in purified hepatic DNA from rats dosed for 3 weeks (5 days/wk) with 3 mg DMN/kg/day (final dose being DMN-^{14}C) (Table 5). The calculated CBI (Lutz, 1979) for DMN was 4119 in hepatic tissue. While a CBI could not be calculated for DX since no DNA alkylation was observed, if it is assumed that this detection limit reflects an upper bounds the CBI for dioxane would be <0.09.

Consistent with the lack of in vivo genotoxicity, DX was also negative

in the Ames' Salmonella mutagenesis assay (Table 6). No increase in the background reversion rate was observed with or without a metabolic activating system over a broad range (0 to 103 mg DX/plate) using S. typhimurium strains TA 1535, 1537, 1538, 98 and 100. The top dosage used was the maximum amount of pure DX possible per assay (0.1 ml/plate) and was bacteriostatic in assays without metabolic activation. Primary rat UDS assays of DX were also negative.

DISCUSSION

The objective of this study was to elucidate the likely mechanism by which DX and HCBD produce hepatic and renal neoplasms, respectively. In general, tumorigenic compounds appear to form a continuum between those substances causing tumors primarily through a direct interaction with cellular DNA (genetic) and those acting primarily through other indirect means (nongenetic or epigenetic) (Watanabe, et al, 1980; Reitz, et al., 1980; Schumann, et al., 1980; Williams and Weisburger, 1980).

The genetic mechanism of tumor initiation is best described by the somatic cell mutation theory of chemical carcinogenesis. This theory states that a chemical carcinogen interacts with cellular DNA in such a way as to cause a heritable alteration in the genetic code of the cell at dosages causing little visible cytotoxicity. Following replication, a transformed cell may result which can ultimately lead to tumor formation. Mitigating factors in this progression are the various DNA repair enzyme systems which appear to serve as the cell's primary defense mechanism against somatic mutations. It is the deficiency in these DNA repair enzymes which is believed to cause individuals with Xeroderma pigmentosum to be sensitive to UV radiation giving rise to a high incidence of skin cancer (Cleaver, 1968; 1969; Maher et al., 1976). Conversely in normal cell cultures the momentary halting of cell division has been observed to decrease UV mutagenic activity probably by giving repair mechanisms added time to repair the UV altered DNA molecules (McCormick, 1979). Thus the absence of tumors in animals receiving low doses of genetically acting carcinogens may be explained to some extent by the repair activities of DNA repair enzyme systems.

Chemicals may also produce tumors through numerous other nongenetic or epigenetic mechanisms; one such mechanism is cytotoxicity. In this case, visible tissue damage occurs with little or no direct interaction of the test compound with DNA. The resultant cellular regeneration is accompanied by an increased rate of DNA replication in turn increasing the chance of spontaneous base errors which normally occur during this process, albeit at a low rate (Weymouth and Loeb, 1978). Once replication has occurred, these errors appear to be "fixed" into the genetic code as they go unrecognized by repair systems which have had less time to act on them in the rapidly replicating genome. This process acts with both naturally occurring endogenous and exogenous agents. Evidence for this

has come from the findings of Berman et al., (1978) who observed that increased cell division of normal cells increases their susceptibility to a mutagenic chemical. Consistent with these concepts chronically inflamed or scarred tissues have been found to be more susceptible to cancers in humans (Laroye, 1974). Additionally, repeated tissue damage with a physical agent (dry ice) and resultant regeneration has induced tumors in mice (Berenblum, 1929) and, this process may be similar to the production of local sarcomas following repeated subcutaneous injection of inert sugars in mice and rats (Grasso and Goldberg, 1966).

The carcinogenic risk a chemical may pose to animals varies greatly depending on which mechanism of tumor development is predominant. Primarily genotoxic compounds may produce tumors without producing a significant degree of tissue damage and thus go undetected until a tumor is pathologically evident. In contrast a nongenotoxic compound may be one producing reversible effects (one such effect is cytotoxicity) prior to the formation of a tumor, and the avoidance of high cytotoxic exposures of the later group of chemicals may preclude any risk of tumor development.

DX was not observed to be genotoxic to rats at tumorigenic dose levels. No *in vivo* hepatic DNA alkylation (detection limit of 1 alkylation/ 10^6 nucleotides) nor increase in hepatic DNA repair were observed to occur in rats dosed by gavage with 1000 mg DX/kg. Indeed, DX did not display any mutagenic activity in the Ames' *Salmonella* mutagenicity assay using tester strains capable of detecting either base pair or frameshift mutations. DX was also observed to be negative in the Williams' primary heaptocyte UDS assay.

Despite a lack of genotoxic activity, repeated high dosages of DX given to rats were hepatotoxic. Exposure to 1000 mg DX/kg/day via their drinking water for 11 weeks resulted in elevated liver to body weight ratios, (12%), a small degree of hepatic histopathological change and an enhanced hepatic DNA synthesis (1.5 fold). These changes reflect the extensive cytotoxicity observed histopathologically by Kociba et al. (1974) in rats chronically administered a similar dose level of DX (1015 mg/kg/day) via their drinking water. Chronic exposure resulted in an excess of hepatocellular carcinoma after up to 2 years exposure to DX. A lower dosage of DX (10 mg/kg/day) when administered to rats under the same regime was not observed to be tumorigenic or cytotoxic (Kociba et al., 1974). Neither did this dose level produce any changes vs control animals

in the present study. DX thus appears to be a compound causing tumors via a nongenetic mechanism possibly through repeated tissue injury when administered to rats at cytotoxic dosage levels.

An alternate nongenetic mechanism of DX tumorigenicity based upon the phenobarbital-like hepatic mixed-function oxidase (MFO) enzyme induction characteristics of DX, is also plausible. DX has been observed to induce the activities of these enzymes in the hepatic tissues of mice (Pawar and Mungikar, 1976) and rats (this laboratory, unpublished data) repeatedly dosed with high levels of DX. When coupled with the observations of increased rat hepatocyte size and DNA synthesis noted in the present study, and the ultrastructural changes (including degranulation of rough endoplasmic reticulum) observed by Argus et al. (1973) in DX-treated rats, these findings suggest a phenobarbital-like mechanism of tumor development in rats. As reviewed by Tennekes (1979), this mechanism may involve a common event such as degranulation of rough endoplasmic reticulum which would result in alterations in protein synthesis and gene expression (Wright et al., 1977), or simply an enhancment of the expression of pre-existing oncogenic factors due to a generalized hyperplastic response to the chemical. The later theory has been supported primarily by findings using phenobarbital treatment of "high spontaneous" tumor forming mice (Peraino et al., 1973). While a spontaneous background incidence of only 2-3.5% hepatocellular carcinoma in male Sprague-Dawley rats has occasionally been observed in our laboratory, this oncogenic potential may have been promoted by the hyperplasia induced by high dosages of DX.

Most important is the evaluation of potential carcinogenic risk associated with a chemical operating solely by a nongenetic mechanism. In direct contrast to a genotoxic carcinogenic mechanism a chemical functioning by a nongenetic mechanism typically portrays the characteristics of reversibility and threshold. Specifically, carcinogenic risk is associated only with dose levels which induce the physiologic and/or toxicologic manifestation, and these pharmacologic effects generally show threshold doses. Consistent with these characteristics it is noteworthy that DX-induced hepatic carcinogenesis occurs only at those high doses which also induce MFO enzyme induction and hepatotoxicity; at lower doses, neither these toxicologic/pharmacologic effects nor carcinogenicity occur.

Unlike DX, HCBD was observed to exhibit a small degree of genotoxicity. Renal genotoxicity was observed by the occurence of a relatively small amount of renal DNA repair (1.27 to 1.54 fold increases) and alkylation (0.78 alkylations/10^6 nucleotides) in rats administered a tumorigenic dose level of 20 mg HCBD/kg. This later observation appeared to be confirmed by HPLC separation of a HCBD-DNA adduct. However, another measure of genotoxic potential, the Ames' bacterial mutagenesis assay, was negative utilizing target cells sensitive to base pair substitution and some frameshift mutagens (TA 100). These mutagenesis findings are at variance with those of Simmon (1977) who reported HCBD to be a potent mutagen in S. typhimurium TA 100 with and without metabolic activation. Supportive of the present assay findings was the negative response of HCBD in the UDS bioassay. As no purity information was reported by Simmon (1977), the possibility that another component or a contaminant of the HCBD formulation used was responsible for the reported mutagenicity of HCBD cannot be ruled out.

HCBD related renal cytotoxicity was also observed in rats. Upon repeated exposure of rats to 20 mg HCBD/kg/day via gavage for 3 weeks, a 1.8 fold increase in renal DNA synthesis activity and a moderate amount of histopathology were noted. Yet, after repeated exposure to nontumorigenic dose levels of 0.2 mg HCBD/kg/day, no changes in the basal renal DNA synthesis rate or microscopic degenerative changes in renal tissue occurred. These findings agree with those of Kociba et al. (1977) in which lifetime ingestion of 20 mg HCBD/kg/day by Sprague-Dawley rats resulted in a considerable degree of renal tissue damage and an increased incidence of renal neoplasms relative to controls. At a 0.2 mg HCBD/kg/day dosage level, no pathological changes in renal tissue (including tumors) vs controls were observed.Thus, the observation of significant renal cytotoxicity of high dosage levels of HCBD to rats suggest that HCBD may be inducing tumors by primarily a nongenetic mechanism. Yet, the presence of a very small degree of HCBD genotoxicity places this compound in an intermediate position between those compounds causing tumors solely via a genetic or nongenetic mechanism.

The findings for DX and HCBD are in sharp contrast to those observed using the potent genotoxic agent DMN. Genotoxicity was evidenced by large increases in DNA repair ratios and high levels of DNA alkylation in rats repeatedly administered a carcinogenic dose level of 3 mg DMN/kg

for 3 weeks. HCBD by comparison, produced only 1/2 the renal DNA repair and only 1/36th the renal DNA alkylation that DMN did at tumorigenic dose levels. Indeed, if DNA alkylation values are normalized for dose and the renal covalent binding index (Lutz, 1979) of the two compounds compared, 587 for DMN vs 10.2 for HCBD, the genotoxic potential of HCBD appears quite small. Unlike DX and HCBD, the absence of any significant increase in hepatic or renal DNA synthesis and histopathology in rats dosed repeatedly with a tumorigenic dosage of DMN relative to controls indicated a general lack of cytotoxicity.

In summary, the lack of genotoxicity at tumorigenic dose levels indicate that DX causes hepatocellular carcinoma in rats via a nongenetic mechanism. Like DX, HCBD also appears to cause tumors (renal neoplasms) in rats via primarily a nongenetic mechanism although a minor genotoxic component cannot be ruled out for HCBD. The response of rats exposed to either of these compounds contrasts sharply with the response observed towards the genotoxin, DMN. Thus, tumorigenic risk following exposure to DX or HCBD as dictated by their apparent mechanisms of tumorigenic action in rats is likely to be present only at cytotoxic dose levels .

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TITLE OF STUDY: DIFFERENTIATION OF THE MECHANISMS OF ONCOGENICITY OF 1,4-DIOXANE AND 1,3-HEXACHLOROBUTADIENE IN THE RAT

HET-1.4-29-(7)

In compliance with Good Laboratory Practice Regulations, the study phases were inspected by the Quality Assurance Unit and the results of these inspections reported to Management and the Study Director on the dates listed below. The report accurately reflects the data generated in accordance with the regulations and standard operating procedures of the laboratory. All data and the reports are located at the submitting laboratory.

Study Started: 6-19-79 Report Issued Date: December 23, 1980

Dates of Inspection:	Date of Report:
24 Oct 79	2 Nov 79
28 Jan 80	5 Feb 80
2 June 80	5 June 80
17 Sept 80	25 Sept 80
10 Nov 80	11 Nov 80

W. E. Hoover 23 December 80

W. E. Hoover Date
Quality Assurance
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TABLE 1

Weight gain, liver weight, DNA content, DNA synthesis and histopathology data for Sprague-Dawley rats dosed acutely or repeatedly (11 weeks via drinking water) with saline, 10 mg, 100 mg or 1000 mg dioxane/kg.

Experiment	Wt. Gain g	Liver Wt. / Body Wt. %	µg DNA / g tissue	dpm ^3H[a] / µg DNA	Histopathology[b]
Acute-I[c] Control	40 (5.8)	4.2 (0.1)	2064 (199)	8.68 (1.37)	-
10 mg/kg	45 (5.8)	4.2 (0.2)	2003 (47.3)	12.33 (3.22)	-
1000 mg/kg	22* (12.6)	4.1 (0.2)	1820 (59.4)	7.35 (1.26)	-
Acute-II[c] Control	68.8 (9.46)	4.0 (0.21)	2158 (165)	10.9 (2.24)	-
10 mg/kg	46.3* (10.3)	4.4 (0.31)	2167 (80.0)	9.26 (1.54)	-
100 mg/kg	41.3* (10.3)	4.3 (0.15)	2039 (232)	13.5 (1.94)	-
1000 mg/kg	31.3* (4.79)	4.2 (0.21)	2206 (32.8)	10.7 (0.960)	-
Repeated[d] Control	256 (31.8)	3.3 (0.15)	2210 (200)	2.73[c] (0.60)	-
10 mg/kg	279 (19.0)	3.3 (0.29)	2155 (102)	3.36 (0.34)	-
1000 mg/kg[e]	233 (16.9)	3.7* (0.22)	2366 (301)	4.09* (0.65)	+

[a] Thymidine [6-^3H] incorporation

[b] + = hepatic histopathology present
- = hepatic histopathology absent

[c] Mean of 4 animals (± Standard Deviation)

[d] n = 5

[e] n = 6

*Significantly different from control ($p < 0.05$)

TABLE 2

Weight gain, kidney weight, DNA content, DNA synthesis and histopathology data for Sprague-Dawley rats dosed acutely or repeatedly (3 weeks) with corn oil, 0.2 mg or 20 mg 1,3-hexachlorobutadiene/kg.

Experiment[a]	Wt. Gain g	Kidney Wt. / Body Wt. %	µg DNA / g tissue	dpm ^3H[b] / µg DNA	Histopathology[c]
Acute-I					
Control	43.0 (2.74)	0.86 (0.054)	4458 (577)	16.2 (1.46)	-
0.2 mg/kg	44.4 (6.27)	0.79 (0.047)	4166 (399)	14.7 (2.53)	-
20 mg/kg	49.0 (6.52)	0.82 (0.042)	3781 (667)	18.7 (4.15)	-
Acute-II					
Control	55 (6.1)	0.86 (0.034)	4491 (888)	23.0 (2.25)	N
0.2 mg/kg	57 (2.7)	0.86 (.0.022)	4607 (391)	25.4 (2.81)	N
20 mg/kg	55 (6.1)	0.85 (0.051)	5052 (244)	34.8[d]* (4.08)	N
Repeated					
Control	149 (23)	0.77 (0.073)	2740 (384)	6.75 (1.85)	-
0.2 mg/kg	150 (15)	0.76 (0.065)	2719 (174)	6.54 (0.84)	-
20 mg/kg	83* (22)	1.00* (0.088)	2844 (153)	12.1 (4.59)	+

[a] Mean of 5 animals (± Standard Deviation)

[b] Thymidine [6-^3H] incorporation

[c] + = renal histopathology present; - = renal histopathology absent

[d] n = 4

*Significantly different than controls ($p < 0.05$).

N = no histological observation

TABLE 3

Weight gain, organ weight, DNA content, DNA synthesis and histopathology data for Sprague-Dawley rats dosed acutely with saline or 3 mg DMN/kg/day for 3 weeks.

Treatment[a] Group	Wt. Gain g	Organ Wt. / Body Wt. %	µg DNA / g tissue	dpm ^{3}H / µg DNA	Histopathology[b]
Control	88.4 (11.1)				
-Liver		4.04 (0.40)	2079 (514)	1.81 (0.41)	-
-Kidney		0.74 (0.033)	3253 (694)	2.68 (0.16)	-
DMN	86.6 (12.0)				
-Liver		4.02 (0.26)	2495 (442)	2.51 (0.46)	-
-Kidney		0.74 (0.04)	3267 (562)	2.50 (0.46)	-

[a]Mean of 5 animals (± Standard Deviation) given.

[b]+ = histopathology evident; - = no histopathology present relative to controls.

TABLE 4

In Vivo hepatic (DMN, DX) and renal (DMN, HCBD) DNA repair measured in Sprague-Dawley rats dosed with 1000 mg DX/kg (hepatic), 20 mg HCBD/kg (renal) or 20 mg DMN/kg (hepatic and renal).

	dpm/μg DNA[a]					
Treatment	Control	Control plus HU[b]	Treated	Treated plus HU	C.F.[c]	R.R.[d]
DX[e] (Hepatic)	28.6 (6.65)	4.04 (0.642)	30.9 (7.12)	4.38 (0.723)	1.08	1.00
HCBD[f] I (Renal)	41.6 (6.34)	0.602 (0.080)	38.4 (6.06)	0.704 (0.054)	0.923	1.27
II	42.2 (7.83)	0.802 (0.072)	32.9 (10.2)	0.964 (0.129)	0.780	1.54
DMN[g] (Hepatic)	16.5 (2.50)	2.43 (0.470)	9.01 (1.95)	4.93 (0.099)	0.546	3.72
(Renal)	30.4 (4.35)	0.824 (0.176)	16.2 (0.071)	1.26 (0.173)	0.533	2.87

[a] Average of n animals (± Standard Deviation)

[b] HU = Hydroxyurea

[c] Correction factor (C.F.) = $\frac{\text{Treated}}{\text{Control}}$

[d] Repair Ratio (R.R.) = $\frac{\text{Treated plus HU}}{(\text{Control plus HU})(\text{C.F.})}$

[e] n = 4

[f] n = 5

[g] n = 3

TABLE 5

Hepatic (DX, DMN) and renal (HCBD, DMN) DNA alkylation data for Sprague-Dawley rats dosed with either 1000 mg DX-^{14}C, 20 mg HCBD-^{14}C or 3 mg DMN-^{14}C.

Treatment	Detection Limit	Alkylations/ 10^6 nucleotides	CBI[a]
DX (hepatic)[b]	$\geq$ 1.0	ND[c]	<0.09
HCBD (renal)[d]	$\geq$ 0.005	0.78(0.87)[e]	10.2
DMN[f]			
(hepatic)	$\geq$ 6.3	167(20.2)	4119
(renal)	$\geq$ 1.1	23.8(3.14)	587

[a] CBI = Covalent Binding Index (Lutz, 1979)

$$= \frac{\mu\text{moles alkylation/mole nucleotides}}{\text{mmole compound/kg body weight}}$$

[b] n = 2

[c] ND = none detected

[d] n = 8

[e] (± standard deviation)

[f] n = 3

TABLE 6

In vitro Salmonella typhimurium mutagenesis assay of DX. Results shown as number histidine revertants/plate.

Strain	Activation[a] System	Control[b]	Positive[b] Control	DX/plate[b] 5.17 mg	15.5 mg	31.0 mg	62.0 mg	103 mg
TA 1535	+	7.3 (2.9)	75.0[c] (11.1)	8.0 (3.5)	10.0 (5.2)	13.3 (1.53)	13.7 (0.6)	12.3 (4.0)
	-	8.7 (0.6)	1027[d] (64.1)	16.0 (3.6)	11.0 (4.4)	10.7 (1.5)	8.3 (5.0)	6.0[e] (2.6)
TA 1537	+	9.0 (4.0)	148[f] (7.2)	8.7 (2.9)	8.3 (0.6)	5.0 (1.0)	6.0 (2.6)	5.0 (1.7)
	-	6.0 (2.0)	416[g] (26.8)	7.3 (0.6)	5.7 (2.1)	3.7 (0.6)	3.0[e] (2.0)	1.7[e] (1.5)
TA 1538	+	21.3 (4.0)	898[h] (27.7)	15.7 (3.1)	21.3 (7.8)	20.3 (7.1)	22.0 (4.4)	16.7 (3.2)
	-	8.3 (3.2)	615[i] (173)	11.3 (4.5)	10.0 (2.0)	9.0 (4.0)	5.7[e] (2.1)	4.3[e] (1.5)
TA 98	+	28.3 (3.1)	614[h] (154)	38.7 (2.1)	38.3 (9.0)	59.7 (15.7)	37.0 (6.2)	31.3 (2.9)
	-	19.7 (1.5)	417[i] (13.4)	26.3 (7.1)	22.7 (5.7)	30.0 (5.0)	23.0[e] (3.6)	6.7[e] (4.0)
TA 100	+	160.3 (22.2)	469[c] (92.9)	178 (8.6)	149 (17.9)	159 (9.71)	153 (9.54)	161 (16.5)
	-	138 (16.8)	991[d] (63.1)	152 (4.5)	147 (12.0)	138 (2.5)	150 (13.4)	144[e] (15.6)

[a] + = Metabolic activation system present
- = Metabolic activation system absent

[b] Revertants/plate representing mean of 3 assays (± standard deviation)

[c] 2-Anthramine (50 µg/plate)

[d] N-methyl-N'-nitro-N-nitrosoguanidine (50 µg/plate)

[e] Background lawn toxicity observed

[f] 8-Aminoquinoline (100 µg/plate)

[g] Quinacrine mustard (50 µg/plate)

[h] 2-Acetamidofluorene (250 µg/plate)

[i] 2-Nitrofluorene (100 µg/plate)

TABLE 7

In vitro Salmonella typhimurium TA 100 mutagenesis assay of HCBD. Results shown as number histidine revertants/plate.

Activation[a] System	Control[b]	Positive[b] Control	HCBD/plate[b] 416 µg	832 µg	1665 µg	2498 µg	4995 µg
+	138 (13.0)	414[c] (79.2)	121 (5.9)	108 (12.8)	132 (16.0)	107 (12.5)	102 (7.3)
-	150 (18.7)	1298[d] (19.8)	110 (18.6)	89.8 (16.7)	101 (11.2)	90.0 (10.3)	83.0 (16.4)

a + = Metabolic activation system present
  - = Metabolic activation system absent

[b]Revertants/plate representing mean of 4 assays (± standard deviation)

[c]2-Anthramine (50 µg/plate)

[d]N-methyl-N'-nitro-N-nitrosoguanidine (50 µg/plate)