

Effects of Vinylidene Chloride on DNA Synthesis and DNA
Repair in the Rat and Mouse: A Comparative Study
with Dimethylnitrosamine

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macromolecules correlates well with the tumorigenic effects observed by Maltoni *et al.* (1977). Binding of VDC metabolites to intracellular macromolecules is greater in the mouse than in the rat. Furthermore, the highest level of macromolecular binding is observed in the kidneys of male mice, the site where tumors were observed by Maltoni. Since it is a currently popular theory that alkylation of DNA by electrophilic species is a critical event in the induction of neoplasia (Miller and Miller, 1966), one objective of this study was to characterize the interaction of [^{14}C]VDC with nucleic acids *in vivo*.

However, alkylation of DNA to initiate the tumorigenic process is not the only factor to be considered. It is known that certain chemicals and surgical procedures, while apparently not tumorigenic themselves, act to "promote" the induction of tumors from other sources (Van Duuren, 1969; Date *et al.*, 1976). "Promotion" can be significant even if the initial oncogenic stimulus is not known. For example, Peraino *et al.* (1973) suggest that phenobarbital enhances the "spontaneous" rate of tumor formation in mice, while Berenblum (1929) found that repeated freezing of the skin was carcinogenic in mice without exposure to any chemical agent. Berenblum further reported that many types of repeated cell damage can dramatically promote an oncogenic stimulus (Berenblum, 1944).

Although the mechanisms of "promotion" are not well understood, a common characteristic of chemical promoters, partial hepatectomy, and chronic irritation is that increased cell division is present (Marx, 1978). This is reflected by an increase in the amount of normal DNA synthesis as replication takes place. Consequently, in addition to measuring direct interaction of VDC with DNA, we have attempted to assess the "promoter" or epigenetic activity of VDC by measuring the rates of incorporation of [^3H]thymidine into the DNA of various tissues.

The objectives of this work were thus to

investigate the importance of both genetic and epigenetic mechanisms in the induction of tumors by VDC. This knowledge is important for determining whether the observation of tumors in mice exposed to toxic levels of VDC has any relevance for assessing the potential hazard for humans exposed to non-toxic levels of VDC.

METHODS

Animals

Male mice, CD-1 strain and male rats, Sprague-Dawley strain were obtained from Charles River Laboratories. The animals were 18–20 (mice) or 200–250 g (rats) when received, and were acclimated for at least 1 week before use.

Materials

[^{14}C]VDC (0.443 mCi/mmol) was synthesized by New England Nuclear. This material was labeled at both C-1 and C-2 and had greater than 99% radiochemical purity by gas chromatographic analysis. [^3H]Thymidine (C-6 labeling, TdR) was obtained as a stock item from New England Nuclear with a specific activity of approximately 20 Ci/mmol. Nonradioactive VDC (redistilled, 99.95% pure with residual inhibitor content 1–5 ppm monomethylether of hydroquinone) was obtained from the Saran and Converted Products Research Laboratory, 1603 Building, Dow Chemical Company, Midland, Michigan. Dimethylnitrosamine (Gold Label Grade) was obtained from Aldrich Chemical Company. All other chemicals were of reagent grade from commercial suppliers.

Enzymes used in the purification of DNA (chymotrypsin, trypsin, ribonuclease A, and deoxyribonuclease (DN-100)) were purchased from Sigma Chemical Company.

Exposures

Animals were exposed to VDC vapors under dynamic conditions with either a single pass system or a recirculating system, in a 30-liter glass inhalation chamber. In either case, the concentration of VDC was measured by periodically sampling the chamber atmosphere and determining the VDC concentration by gas chromatography. One-milliliter samples of the chamber atmosphere were chromatographed on a 3-ft column of Porapak QS (80/100 mesh) at 150°C. Quantitation was by peak height with a hydrogen flame ionization detector.

In a single pass system, total flow through the chamber was ~10 liters/min and the VDC concentra-

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Effects of Vinylidene Chloride on DNA Synthesis and DNA Repair in the Rat and Mouse: A Comparative Study with Dimethylnitrosamine. REITZ, R. H., WATANABE, P. G., MCKENNA, M. J., QUAST, J. F., AND GEHRING, P. J. (1980). *Toxicol. Appl. Pharmacol.* 52, 357-370. Exposure to vinylidene chloride (VDC) vapor has been reported to induce tumors in mice, but rats are apparently insensitive to this effect of VDC. This species difference has been correlated with the greater capacity of mice to activate VDC to a reactive electrophile which can react with macromolecules. To increase our understanding of the molecular events associated with this species difference, we have investigated the potential of VDC to cause DNA alkylation, DNA repair, and DNA replication in the liver and kidneys of rats and mice. For comparative purposes, the potent carcinogen dimethylnitrosamine (DMN) was also studied. Male Sprague-Dawley rats and CD-1 mice were exposed to 10 and 50 ppm VDC for 6 hr. DNA alkylation after 50 ppm [¹⁴C]VDC was minimal in liver and kidney of both rats and mice (one or two orders of magnitude less than reported for DMN in rats). Similarly, DNA repair in the kidney of mice exposed to 50 ppm VDC was only 38% higher than control values, while DNA repair in the liver of mice injected with 20 mg/kg DMN was elevated 637%. However, tissue damage and increased DNA replication (25 fold) were seen in the kidneys of mice exposed to 50 and 10 ppm VDC. Comparable effects were not seen in the liver of mice exposed to VDC (50 or 10 ppm) or in the liver or kidneys of rats exposed to 10 ppm VDC. Thus an important distinction between DMN and VDC has been demonstrated. Tumorigenic doses of DMN produced relatively little tissue damage, but were associated with a high degree of DNA alkylation and DNA repair synthesis. In contrast, exposure to tumorigenic doses of VDC resulted in massive tissue damage but induced minimal DNA alkylation or DNA repair synthesis. This suggests that the tumors observed in mice exposed to VDC arise primarily through effects of the chemical on nongenetic components of the cells. Consequently protection of humans from levels of VDC sufficient to cause tissue damage should also serve to preclude any carcinogenic activity of VDC.

Vinylidene chloride (1,1-dichloroethylene, VDC) is an intermediate in the synthesis of many commercially important plastics. Although carcinogenicity of this material has not been observed in several long-term studies with rats (Viola and Caputo, 1977; Rampy *et al.*, 1977; Maltoni *et al.*, 1977),

VDC-related tumors have been reported in mice (Maltoni *et al.*, 1977). Maltoni reported that male mice were far more sensitive to VDC than females, that the target organ in male mice was the kidney, and that the tumorigenicity of VDC was associated with significant injury to kidney tissue in male mice (Maltoni *et al.*, 1977).

This study was funded by the companies supporting the vinylidene chloride projects being administered by the Manufacturing Chemists Association, Washington, D.C.

Studies by McKenna *et al.* (1977) have shown that the metabolism of VDC to electrophilic species which bind to cellular

Isolation of Nucleic Acids

DNA, RNA, and protein were quantitated by the methods of Burton (1956), Brown (1946), and Lowry *et al.* (1951), respectively.

DNA was isolated by a modification of the method of Marmur (1961). Tissues were rapidly excised and frozen on dry ice. The frozen tissues were then homogenized in 0.04 M Tris, 0.015 M NaCl (pH 8.0) to give a final concentration of 5×10^{-2} (w/v). The cells were then immediately lysed by the addition of 0.1 vol of 22% sodium dodecyl sulfate and mixed with 0.2 vol of 5 M NaClO₄. The mixture was heated to 60°C for 10 min, cooled, and extracted with 1.0 vol of 4% isoamyl alcohol in chloroform (1 hr shaking at 20–25°C). After extraction, the aqueous layer was carefully removed. DNA was spooled on a glass rod after addition of 2 vol cold 95% ethanol.

If the DNA was from a [³H]thymidine injected animal, it was blotted dry and then incubated in a minimal amount of DNase (0.1 mg/ml enzyme in 0.05 M phosphate, ~ 0.01 M MgCl₂, pH 7.0) at 37°C until it had dissolved. Aliquots of this solution (hydrolyzed DNA) were counted directly or analyzed by the diphenylamine reaction as described by Burton (1956).

DNA from animals exposed to [¹⁴C]VDC was subjected to further purification. After spooling, the DNA was redissolved in 1 ml of distilled water. 3 ml of original homogenate (gentle agitation overnight at 20–25°C). After dissolution, 0.1 vol of crystalline α -amylase (1 mg/ml dissolved in 0.1 M PO₄, pH 7) were added and the solution was incubated for 30 min at 37°C.

Next an additional 0.1 vol of a solution of trypsin (0.5 mg/ml) and chymotrypsin (0.5 mg/ml) in 0.1 M PO₄ (pH 7) was added and incubation was continued for 90 min. These enzymatic digestions degrade residual glycogen and protein in the DNA preparation. After incubation, 0.2 vol of 5 M NaClO₄ was added and the nucleic acids were spooled as before by the addition of 2 vol of 95% ethanol.

After redissolving in distilled water, DNA and RNA were separated as follows: 0.1 vol of ribonuclease A (0.5 mg/ml in 0.1 M PO₄, 0.01 M citrate, pH 7) was added and incubated at 37°C for 2 hr. DNA was separated from the hydrolyzed RNA by precipitation with 0.25 vol of 25% trichloroacetic acid (TCA) and collected on a Millipore filter.

For assay, the DNA precipitate was dissolved in a minimal volume of 0.1 mg/ml DNase in 0.05 M PO₄ (pH 7.0) also containing 0.01 M MgCl₂. When the DNA was completely hydrolyzed, aliquots were sampled for scintillation counting or colorimetric analysis.

Histopathological Assessment

Samples of kidney and liver were removed at necropsy and fixed in buffered 10% formalin. The tissues were

processed by routine histologic procedures. Sections (5–6 μ m) were stained with hematoxyline and eosine and examined by light microscopy.

Scintillation Counting

Samples of up to 2.5 ml were counted in 15 ml ACS (Amersham) on a Nuclear Chicago scintillation counter (Mark II or Mark III) with quench correction.

Statistical Analysis

I. DNA synthesis. Specific radioactivities (dpm/mg DNA) were calculated for each tissue of each animal. Each treatment tissue group was then analyzed for a significant increase over the appropriate control by use of Student's *t* test. Results were considered significant at a *p* value ≤ 0.05 .

II. [¹⁴C]VDC alkylated DNA. In order to obtain sufficient material for analysis, tissues from five to seven animals were pooled for determination of the specific radioactivity of DNA. Next the logarithm of the specific radioactivity (dpm/mg DNA) was subjected to linear regression analysis versus time (hr) after the termination of the 6 hr exposure to VDC.

III. DNA Repair. Ninety-five percent confidence intervals on the correction factor were calculated after dividing individual treatment values by the mean of the appropriate control group. Specific radioactivities of the DNA from individual animals in the subgroup receiving [³H]thymidine and hydroxyurea after treatment are divided by the correction factor and compared to individual specific radioactivities in the corresponding control subgroup using one-sided *t* test. Increases are considered significant at the *p* = 0.05 level.

IV. DNA prelabeled with [³H]thymidine. Data from control and VDC 50 ppm exposed groups at 0, 48, and 120 hr postexposure were subjected to a two-way, completely random analysis of variance with *p* = 0.05 chosen as the level of significance.

RESULTS

Genetic Effects

The levels of radioactivity which were incorporated into DNA purified from the tissues of animals exposed to [¹⁴C]VDC are summarized in Table I. VDC alkylation of DNA was very low in kidney and liver of both rats and mice. The highest level of alkylation was observed in the kidneys of mice exposed to 50 ppm VDC and cor-

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tion was maintained by metering concentrated VDC
vapor (1000–5000 ppm) from a separate supply bag.
The supply bag was prepared by passing 100 liters of
air (measured with a DTM-115 Singer gas meter) through
a U-tube containing 0.4 to 2.0 ml of VDC so that
the VDC evaporated and mixed thoroughly with the air.

For the recirculating chamber exposures, air was
circulated through the system at 20–30 liters/min with
a Thomas Model 907CA18TF-E pump. Water and CO₂
were removed from the system by Drierite and Ascarite
traps (1 lb of each) in series with the pump. O₂ was
metered into the closed system to replace the CO₂ using
a Manowatch constant pressure valve (R. Cheltenham
Pa.). [¹⁴C]VDC was injected into the system by remov-
ing 10 ml portions of the headspace from a sealed
septum vial (5 ml capacity, containing 2–3 ml of 5%
[¹⁴C]VDC in xylene. Before removing the headspace,
the septum vial was momentarily warmed to 60°C by
immersion in a hot water bath. Because of the large
differences in the vapor pressure of VDC and xylene,
the amount of xylene transferred to the chamber by
this procedure is less than 2% of the amount of VDC
added.

All inhalation exposures were 6 hr in duration and
were initiated between 9 AM and 12 noon. Both control
and exposed animals were deprived of food and water
during the exposure. Otherwise these were available
ad libitum.

DNA Synthesis (Cell Division)

DNA synthesis was measured 48 hr after exposure.
In this procedure, a population of animals was divided
into a control and treated group.

Each group received an ip injection of [³H]thymidine
(500 or 1000 µCi/kg body wt) in a volume of 0.01 ml/g
of body wt. Four hours after the [³H]thymidine injec-
tion, the animals were sacrificed and DNA was isolated
from kidney and/or liver as described elsewhere.

Increased DNA replication was calculated by divid-
ing the mean specific radioactivity of DNA prepara-
tions from treated groups by the mean specific radio-
activity of the DNA preparations from the control
group. It was noted that the degree of labeling of DNA
with [³H]thymidine was relatively constant within a
matched group of animals (i.e., animals with a given
birth date, taken from the same shipment and housed
identically until use) but could vary to two- or threefold
between different shipments of animals. Consequently,
each experiment includes a matched control group and
the ratio of incorporation in treated and control
groups is calculated as a "replicative index." A value
of 1.0 or less indicates that the treatment did not
stimulate DNA replication, while numbers larger than
1.0 show stimulation of DNA replication, indicating
regeneration following cellular injury.

DNA Repair

DNA repair was measured according to the following
procedure. Animals from one population were divided
into treated (T) or control (C) groups. Each group was
further divided into two subgroups (three to six ani-
mals/subgroup). One subgroup received [³H]thymidine
(0.01 ml/g body wt, 500 or 1000 µCi/kg, 20 Ci/mmol
ip) and the other subgroup received an identical injec-
tion of [³H]thymidine dissolved in 40 mg/ml hydroxy-
urea. Four hours later, the animals were sacrificed
by cervical dislocation, and the DNA was prepared
from tissues as described elsewhere.

Repair was calculated from these groups as sug-
gested by Artellini *et al.* (1978). In this procedure
hydroxyurea was administered simultaneously with
[³H]thymidine. This suppresses normal replicative DNA
synthesis, but does not inhibit DNA repair systems
(Cleaver, 1969). This allows more sensitivity for the
detection of the relatively small amount of DNA repair
synthesis. In addition, a treated and control group
injected with [³H]thymidine but not hydroxyurea were
included in each experiment. Evaluation of the data
from the four groups allows calculation of the repair
ratio as follows:

(1) The specific radioactivities of the DNA isolated
from each tissue were calculated (dpm/mg DNA).

(2) Next a correction factor (CF) for the effect of
the test chemical on normal replicative DNA synthesis
was calculated by dividing the mean specific radioac-
tivity of the DNA in the treated group by that of the
control group.

(3) Finally the repair ratio (RR) was calculated as

$$RR = \frac{(T)_{th}}{(C)_{th} \cdot CF}$$

where (T)_{th} = the mean specific radioactivity of the
DNA isolated from the treated subgroup injected with
[³H]thymidine plus hydroxyurea; (C)_{th} = the mean
specific radioactivity of the DNA isolated from the
control subgroup injected with [³H]thymidine plus
hydroxyurea; and CF is the correction factor calculated
in (2).

Repair synthesis of DNA is maximal within a few
hours after treatment, when absorption, distribution,
and (in the case of procarcinogens) metabolic activa-
tion have taken place. Consequently, repair measure-
ments were carried out 4 hr after ip injection of DMN.
In the case of VDC exposure, animals receive the
material throughout the 6-hr exposure chosen to ap-
proximate occupational exposures. Consequently,
metabolic activation of material inhaled early in the
exposure has proceeded for nearly 6 hr by the termina-
tion of the exposure. Therefore repair measurements
were begun immediately after removing the animals
from the chamber.

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TABLE 2
SPECIFIC RADIOACTIVITY OF [³H]THYMIDINE-LABELLED DNA IN MICE WITH AND WITHOUT
EXPOSURE TO 50 ppm UNLABELED VDC (6 hr)^a

Tissue	Specific radioactivity of DNA (dpm/mg DNA, mean $\pm$ SE)		
	Time post-VDC exposure (hr)		
	0	48	120
Kidney			
Control	11.6 $\pm$ 1.8 (n = 4)	15.2 $\pm$ 1.0	12.1 $\pm$ 2.1
VDC (50 ppm) ^c	16.4 $\pm$ 0.99	17.9 $\pm$ 1.3	15.5 $\pm$ 0.72
Liver			
Control	13.8 $\pm$ 4.6 (n = 4)	17.3 $\pm$ 1.9	18.7 $\pm$ 7.6
VDC (50 ppm)	17.4 $\pm$ 3.4	15.3 $\pm$ 3.2	11.4 $\pm$ 2.4

^a Animals were injected with [³H]thymidine 3 days prior to the start of the experiment in order to label their DNA. Then half the animals were exposed to 50 ppm VDC vapor (nonradioactive) for 6 hr, while the remaining animals were held as a control. Then the animals were sacrificed at the indicated times and the specific radioactivity of the DNA in various tissues was determined as outlined under Methods. Unless otherwise indicated, n = 5 in all groups.

^b Data were analyzed by a two-way analysis of variance, completely randomized design. There were no significant time effects and no significant interactions between time and treatment, but a significant ($p < 0.01$) treatment effect was found in the labeling of kidney DNA.

^c Significantly different from control, $p < 0.01$.

dpm/mg DNA immediately following exposure had fallen to 4.3 dpm/mg 96 hr later.) However, calculation of the kinetic parameters following the 10 ppm exposure was not possible because of insufficient data.

The loss of ¹⁴C from the DNA preparations of mice exposed to 50 ppm VDC appeared to be biphasic. An initial rapid phase was noted during the first 8 hr following exposure, with a slower, log-linear phase continuing for at least 192 hr. (Apparent half-lives were calculated from the second phase.)

Another experiment was carried out to establish that cell necrosis produced by VDC had not contributed to the previously observed loss of radioactivity from the DNA of animals exposed to [¹⁴C]VDC (Fig. 1). In this additional experiment DNA was labeled 48 hr prior to treatment by injection of [³H]thymidine. The specific radioactivity of DNA isolated from the kidneys of mice previously labeled with [³H]thymidine was relatively constant, either with or without exposure to 50 ppm VDC (Table 2). The

specific radioactivity of DNA from the livers of these animals also appeared fairly constant (Table 2), but a higher degree of variation in this tissue made it difficult to assess this point precisely. A two-way analysis of variance indicated no significant time effects or interactions, but a small treatment effect was noted in the kidney (~20% increase in specific radioactivity, Table 2).

DNA repair was measured directly in animals exposed to VDC (50 and 10 ppm) or injected with DMN (20, 10, and 3 mg/kg, ip). DMN treatment caused a dramatic increase in the level of hydroxyurea-resistant [³H]thymidine incorporation. The relative increase in repair synthesis caused by DMN, after correction for the effects of DMN or nonrepair synthesis as outlined under Methods, was more than sevenfold, and this increase was dose related (Table 3). In contrast, a much smaller increase in the repair ratio (1.00 $\rightarrow$ 1.38) occurred after exposure of mice to VDC. The only statistically significant increase caused by VDC was that oc-

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After exposure to either 50 or 10 ppm
VDC, the DNA from the liver of mice con-
tained 5- to 10-fold less radioactivity than
the DNA from the kidneys (Table I). In rats
exposed to 10 ppm VDC, the DNA of kidney
contained about twofold greater activity than
the DNA of liver. Compared to mice exposed
to 10 ppm VDC, about sixfold less radioac-
tivity was found in the DNA of rat kidneys,
while similar amounts were found in the DNA
from the liver of both species (Table I)

TABLE I
DNA DAMAGE PRODUCED BY VINYLIDENE CHLO-
RIDE (VDC) AND DIMETHYLNITROSAMINE (DMN) IN
RATS AND MICE^a

Treatment tissue	dpm/mg DNA	Alkyla- tions nucleotide ($\times 10^3$)
50 ppm VDC (mouse)		
Kidney	88 (<i>n</i> = 1)	30
Liver	18 (<i>n</i> = 1)	6.1
10 ppm VDC ^b (mouse)		
Kidney	34 $\pm$ 13.4 (<i>n</i> = 2)	11
Liver	2.80 $\pm$ 0.0 (<i>n</i> = 2)	0.94
10 ppm VDC (rat)		
Kidney	5.9 $\pm$ 1.9 (<i>n</i> = 3)	2.0
Liver	2.6 $\pm$ 1.9 (<i>n</i> = 6)	0.87
10 mg/kg DMN (rat)		
Liver	—	3000-4000 ^c

^a Animals were exposed to [¹⁴C]VDC vapor for 6 hr
at the indicated concentrations. Then they are im-
mediately sacrificed for analysis. Data are expressed
as mean specific radioactivity $\pm$ SD of DNA isolated
from the pooled tissues of five (50 ppm mice), seven
(10 ppm mice), or two animals (10 ppm rats). The
results are the average of multiple determination,
except for the data of 50 ppm VDC mouse. These
animals were part of a larger experiment to determine
the time course of DNA repair in animals exposed
to 50 ppm VDC, and this particular time point was
only determined once.

^b Data from Pegg and Hui (1978).

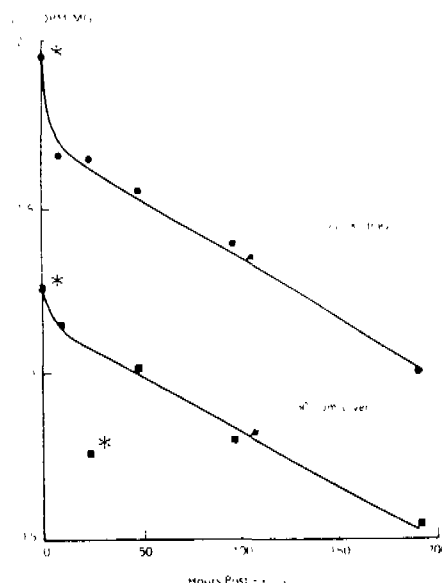


FIG. 1. Time course for radioactivity in the DNA
of CD-1 mice exposed to [¹⁴C]VDC. Animals were
exposed to 50 ppm [¹⁴C]VDC for 6 hr. At the indicated
times, the animals were sacrificed and DNA was
isolated from the pooled tissues of five mice/time point.
Hydrolyzed DNA (5-20 mg) was counted until at
least 10⁴ disintegrations had been accumulated. In each
case, the counting rate was at least twice background.
Half-lives were calculated from linear regression
analysis, and were 83 (kidney) and 91 hr (liver). Half-
lives were calculated from the terminal log-linear
phase of the curves. One point was eliminated from
the regression analysis for liver DNA (24-hr point)
after it was found that the observed point differed
significantly from the predicted value for that time (*p*
= 0.015, one-sided *t* test). The coefficients for the cal-
culated regression line, $\log(\text{dpm/mg DNA}) = a - b$
(time in hr) were: *a* = 1.72, *b* = 0.00364 $\pm$ 0.000345
(kidney) and *a* = 1.16, *b* = 0.00329 $\pm$ 0.000561 (liver).
Correlation coefficients for the linear regressions are
0.9967 (kidney) and 0.9928 (liver).

^c Point not included in regression analysis.

The radioactivity in the DNA from the
liver and kidney of mice exposed to [¹⁴C]-
VDC is rapidly eliminated (Fig. 1). The ap-
parent half-lives were 83 and 91 hr for kidney
and liver, respectively (50 ppm VDC ex-
posure). Radioactivity in the DNA from the
kidney of mice exposed to 10 ppm VDC
appeared to be eliminated more rapidly (30.2

TABLE 4
DNA SYNTHESIS IN TISSUES OF VINYLIDENE CHLORIDE (VDC) AND DIMETHYLNITROSAMINE (DMN) EXPOSED RATS AND MICE (TREATED/CONTROL)^a

Dose/tissue	Mean spec act $\pm$ SE	Ratio
VDC (50 ppm) (mice)		
Kidney	272 ± 15.2 ($n = 4$) 11.0 ± 2.63 ($n = 2$)	24.7 ^b
Liver	26.9 ± 5.40 ($n = 4$) 11.0 ± 3.0 ($n = 2$)	2.45
VDC (10 ppm) (mice)		
Kidney	95.0 ± 15.2 ($n = 5$) 12.3 ± 2.41 ($n = 5$)	7.72 ^b
Liver	17.6 ± 2.96 ($n = 6$) 14.7 ± 1.95 ($n = 5$)	1.20
VDC (10 ppm) (rats)		
Kidney	23.8 ± 3.7 ($n = 3$) 10.8 ± 3.8 ($n = 3$)	2.20 ^b
Liver	16.3 ± 0.87 ($n = 3$) 18.6 ± 2.3 ($n = 3$)	0.88
DMN (20 mg/kg) (mice)		
Liver	(Died before 48 hr)	
DMN (10 mg/kg) (mice)		
Liver	94.0 ± 30.1 ($n = 3$) 47.8 ± 17.6 ($n = 4$)	1.97
DMN (3 mg/kg) (mice)		
Liver	54.5 ± 11.0 ($n = 3$) 47.8 ± 17.6 ($n = 4$)	1.14

^a DNA synthesis was estimated by determining the specific radioactivity of DNA following [³H]thymidine ([³H]TdR) injection. [³H]TdR was injected 48 hr after the treatment suspected of causing cytotoxicity. A positive response is indicated by an increased rate of incorporation of [³H]TdR into DNA relative to a control group, resulting in a ratio greater than 1.0.

^b Ratio significantly greater than one ($p < 0.05$, t -test).

procedure differs from that employed to measure DNA repair in two important respects:

(1) Hydroxyurea is not used to suppress normal DNA replication.

(2) [³H]Thymidine is injected approximately 48 hr after the event suspected of causing tissue damage.

The results of these experiments are summarized in Table 4. In each case, the incorporation of ³H into DNA in the treated group is compared to the incorporation of an untreated control group. Exposure to 50 ppm VDC caused about a 25-fold increase in incorporation of [³H]thymidine into DNA of mouse kidneys, while exposure to 10 ppm VDC produced about an 8-fold increase in [³H]thymidine incorporation in this tissue (Table 4). These effects probably reflect increased levels of DNA replication rather than changes in the DNA precursor pool sizes, since a large number of mitotic figures were seen in treated, but not control, tissues during microscopic examination (Table 5).

Effects on DNA replication in the mouse liver were much smaller: 2.4 and 1.2 times control at 50 and 10 ppm VDC, respectively (Table 4). DMN treatment also produced only a small increase in replication of DNA in the livers of treated mice: 1.1 and 2.0 times control at 3 and 10 mg/kg, respectively.

Rats exposed to 10 ppm VDC exhibited much smaller effects on DNA replication than mice exposed to the same concentration. In the rat kidney DNA replication was only elevated 2.2-fold while DNA replication in the liver was slightly decreased.

DISCUSSION

One of the most popular theories of chemical carcinogenesis is that alkylation of DNA by reactive molecules induces somatic mutations which permanently transform target cells into malignant clones. This theory is supported by the finding that many potent carcinogens have been shown to cause covalent alkylation of various sites in the DNA molecule. For example, Pegg and Hui (1978) found that guanines in the DNA isolated from the liver of rats injected with DMN (10 mg/kg ip) were methylated to the extent of 0.3–0.4% (3000–4000 mol of

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50 ppm VDC, or in the kidneys or livers of
mice exposed to 10 ppm VDC (Table 3).

Epigenetic Effects

A second procedure which measures pri-
marily DNA replication was used to evaluate
nongenetic effects of VDC exposure. This

TABLE 3

DNA REPAIR IN TISSUES OF MICE EXPOSED TO DIMETHYLNITROSAMINE (DMN) OR VINYLIDENE
CHLORIDE (VDC), RATIO OF TREATED ANIMALS TO CONTROLS^a

Dose/tissue	(A) [³ H]TdR only (Mean spec act ± SE (treated/control))	(B) Correction factor (95% confidence limit)	(C) [³ H]TdR + HU (Mean spec act ± SE) (treated/control)	(D) Repair ratio [(C) divided by (B)] (observed/ expected, 95% confidence limit)
VDC-50				
Kidney	5.30 ± 1.15 ($n = 4$) 9.45 ± 2.28 ($n = 3$)	0.561 ± 0.285^b	1.74 ± 0.0488 ($n = 4$) 2.25 ± 0.0917 ($n = 4$)	1.38 ± 0.090^b
Liver	9.69 ± 1.50 ($n = 4$) 12.9 ± 1.63 ($n = 4$)	0.751 ± 0.275	16.0 ± 2.13 ($n = 4$) 18.4 ± 0.434 ($n = 4$)	1.16 ± 0.363
VDC-10				
Kidney	14.7 ± 1.20 ($n = 6$) 14.6 ± 1.86 ($n = 6$)	1.01 ± 0.164	2.67 ± 0.197 ($n = 3$) 2.27 ± 0.640 ($n = 4$)	1.16 ± 0.255
Liver	17.2 ± 2.36 ($n = 6$) 15.6 ± 1.28 ($n = 6$)	1.10 ± 0.305	2.16 ± 0.195 ($n = 3$) 2.57 ± 0.378 ($n = 4$)	0.764 ± 0.222
DMN (20 mg/kg)				
Liver	34.4 ± 1.03 ($n = 3$) 80.5 ± 1.92 ($n = 3$)	0.427 ± 0.038^b	44.7 ± 4.51 ($n = 3$) 14.2 ± 2.64 ($n = 3$)	7.37 ± 2.16^b
DMN (10 mg/kg)				
Liver	27.0 ± 2.94 ($n = 4$) 41.6 ± 14.5 ($n = 4$)	0.649 ± 0.166^b	15.5 ± 1.95 ($n = 4$) 7.85 ± 1.28 ($n = 3$)	3.04 ± 0.896^b
DMN (3 mg/kg)				
Liver	19.0 ± 1.41 ($n = 6$) 25.0 ± 4.93 ($n = 6$)	0.760 ± 0.114^b	9.57 ± 2.32 ($n = 3$) 7.85 ± 1.28 ($n = 3$)	1.60 ± 1.14

^a Animals were injected with [³H]thymidine ([³H]TdR) or [³H]TdR plus hydroxyurea (HU). HU is given at the same time as [³H]TdR in order to block most of the replicative DNA synthesis. The times of [³H]TdR or [³H]TdR + HU injection were (a) immediately following a 6-hr inhalation exposure to VDC, or (b) 4 hr after ip administration of DMN. The amount of DNA repair is reflected in column (D) where the "repair ratio," calculated as outlined under Methods, is presented. A value of 1.0 in this column indicates little or no repair is present, while any increase in this value above 1.0 is taken as indication that prior DNA damage has occurred.

The repair ratio is the ratio of the observed hydroxyurea-resistant TdR incorporation to that which would be expected from consideration of corrected control values. It is calculated as (C) divided by (B) as outlined under Methods.

^b Ratio is significantly different than 1.0, t test with $p < 0.05$.

^c Animals received 1000 μ Ci/kg [³H]thymidine instead of the 500 μ Ci/kg used in the rest of the experiment.

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that the mutagenic effects of this chemical will be much less than those produced by a potent *in vivo* alkylator such as DMN.

Since the determination of radioactivity in the DNA preparations of these animals was used to indicate the degree of genetic damage, it is important to eliminate the possibility that this radioactivity could arise from sources other than DNA alkylation. Two such sources might be: (1) contamination of the DNA preparation with other macromolecules which are radioactive (e.g., protein), and (2) breakdown of [¹⁴C]VDC by metabolism with subsequent incorporation of radioactivity into DNA from the C-1 pool (Gillette and Pohl, 1977).

Contamination of DNA

To eliminate contamination as a source of radioactivity, the DNA preparation was extensively purified. In addition to the basic procedure of Marmur (1961), the DNA was treated with purified enzymes which specifically degraded protein (trypsin and chymotrypsin treatment), glycogen (α -amylase treatment), and ribonucleic acid (ribonuclease A treatment). Analysis of the final preparation of DNA indicated that it contained less than 0.6% protein and less than 2% RNA by weight. The high purity of the DNA preparations makes it unlikely that contamination is responsible for the radioactivity observed.

Incorporation of C-1 Fragments

If the majority of the radioactivity in the DNA prepared from animals exposed to [¹⁴C]VDC had arisen from incorporation of radioactive C-1 fragments, then it should appear in normal (i.e., nonalkylated) DNA bases. Consequently, this radioactivity should be very persistent. (Gross *et al.* (1969) report a $t_{1/2}$ of >30 days for liver DNA). However, if the radioactivity in the DNA has arisen by alkylation, DNA repair

systems should cause it to be lost much more rapidly. In order to investigate this point, DNA was isolated from [¹⁴C]VDC exposed animals at various times after exposure. As shown in Fig. 1, this radioactivity was rapidly eliminated, with half-lives of less than 100 hr. Cell necrosis could not be responsible for this loss since it was shown in another experiment that exposure to the same concentration of VDC (50 ppm) did not cause the turnover of normal DNA previously labeled with [³H]thymidine (Table 2). Since 70–80% of the radioactivity was lost in the time period studied (192 hr), it appears that most of the radioactivity found in DNA after exposure to [¹⁴C]VDC is recognized as "damage" by the DNA repair systems, and consequently represents direct alkylation rather than incorporation of C-1 fragments. The functioning of DNA repair mechanisms in response to this small amount of damage is a significant observation, because these repair mechanisms are thought to play an important role in protecting organisms from small amounts of genetic damage.

In order to carry out a direct comparison of the genetic effects of DMN and VDC in the mouse, a second experimental procedure for assessing DNA repair *in vivo* (as an indicator of preceding DNA damage *in vivo*) was developed. [³H]thymidine was used to measure *in vivo* DNA synthesis in the presence of hydroxyurea (400 mg/kg) (see Methods).

DMN treatment caused a dramatic increase in the level of HU-resistant [³H]-thymidine incorporation (Table 3). The ratio of observed/expected incorporation (Repair Ratio, Column D in Table 3) was elevated more than sevenfold at the highest dose of DMN (20 mg/kg) and declined in a dose-related fashion. In contrast, VDC exposure caused only a slight increase in this ratio (1.00 $\rightarrow$ 1.38) in the kidneys of mice exposed to 50 ppm. The "repair" ratio in the livers of these animals, or the livers or kidneys of mice exposed to 10 ppm of VDC, was not

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TABLE 5

SUMMARY OF HISTOPATHOLOGY FINDINGS IN TISSUES FROM MICE TREATED WITH VINYLIDENE
CHLORIDE (VDC) OR DIMETHYLNITROSAMINE (DMN)^a

Exposure	Postexposure (hr)	Histologic findings	
		Kidneys	Liver
VDC (50 ppm), 6 hr	0	Toxic nephrosis	Slight centrilobular swelling
	8	Progressing nephrosis	
	24	Progressing nephrosis	
	48	Increased mitotic figures	No effect
	96	Regeneration apparent	
	192	Regeneration continuing	
VDC (10 ppm), 6 hr	0	Slight dilation, swelling	
	96	Nephrosis-variable 0-20% affected	
DMN (10 mg/kg ip)	4		Centrilobular swelling Hyaline degeneration
	52		Accentuated lobular pattern, necrosis minimal or absent
DMN (3 mg/kg ip)	4		Centrilobular swelling
	52		No effect

^a Three to six animals were given the indicated treatment and then sacrificed at the appropriate time. Tissue slices were prepared by standard histochemical procedures

methylated base/10⁶ mol of guanine). In order to see whether VDC inhalation produced similar effects, groups of rats and mice were exposed to [¹⁴C]VDC vapor, and the DNA from various tissues of these animals was isolated and purified.

The most striking observation in these studies was the extremely low degree of alkylation detected following [¹⁴C]VDC exposure. For direct comparison with the data of Pegg and Hui (1978), the degree of alkylation of DNA is calculated from the disintegrations per minute observed per milligram DNA and the specific radioactivity of the VDC. Assuming the average molecular weight of a nucleotide residue in DNA is 330, the maximum level of DNA alkylation by VDC was 0.003% or 30 alkylations/10⁶ nucleotides (Table 1). This occurred in the DNA isolated from the kidneys of mice exposed to 50 ppm of [¹⁴C]VDC. When mice were exposed to 10 ppm VDC, about one-third as many alkylations occurred in the

DNA of the kidney. At a site where VDC-related tumors were not observed (mouse liver) or in the kidney or liver of an insensitive species (the rat), alkylation of DNA by VDC was even less. Assuming that the degree of alkylation of guanine is typical of other DNA bases, we see that DMN produced about two orders of magnitude more DNA binding than VDC. The doses of DMN and VDC (10 mg/kg ip, and 50 ppm inhalation, 6 hr) are about the highest levels that can be administered to CD-1 male mice without causing some delayed mortality, and are at least roughly equivalent in terms of their acute toxicity.

There are many potential sites for alkylation of DNA by VDC, and it is almost certain that some of these sites will differ from others in terms of their susceptibility to repair and/or ability to cause mispairing of bases during replication. Nevertheless, the observation that overall *in vivo* alkylation of DNA by VDC is very low is suggestive

results are particularly significant in view of the fact that the mice in Maltoni's study (Maltoni *et al.*, 1977) developed treatment-related tumors in the kidney, but not in the liver. It is also noteworthy that in the male rat, a species which is apparently resistant to the tumorigenic effects of VDC, effects on DNA synthesis were minimal. DNA replication in the kidney of VDC-treated rats (10 ppm, 6 hr) was elevated twofold (Table 4), while VDC treatment had no detectable effect on DNA synthesis in the liver of rats.

DMN was very different than VDC in its effects on DNA replication in mice. After 10 mg/kg DMN, the DNA replication in the livers of treated mice was elevated less than twofold over controls, while in mice receiving 3 mg/kg DMN this parameter was elevated only 14% (Table 4). Since the liver is a target organ for tumor induction after chronic administration of DMN in mice (IARC, 1972), it is appropriate to compare changes induced in this tissue by DMN to changes induced in the kidneys by VDC. When this is done, it is clear that exposure of mice to levels of VDC found by Maltoni to cause kidney tumors causes a dramatic increase in DNA synthesis, while treatment of mice with the known mutagen DMN at a carcinogenic dose failed to elicit a comparable response.

To verify the contention that comparable degrees of tissue damage were not produced by DMN and VDC, tissue samples from treated mice were taken for histopathological assessment. The results are summarized in Table 5. As reported by Maltoni *et al.* (1977), the kidney tissue was severely affected. VDC exposure (50 ppm) produced severe necrosis, and some necrosis was also seen after 10 ppm VDC. Liver tissue was much less sensitive to the effects of VDC. DMN (10 mg/kg) produced some histopathology in the liver, but it is noteworthy that this was not a necrotic type of reaction. The kidney damage produced by 50 ppm of VDC showed progressive necrosis

for the first 24 hr with regeneration and recovery beginning at 48 hours, and continuing throughout the 192-hr period. Treatment related effects were still apparent in the kidney at 192 hr postexposure, but a significant recovery had occurred at this time.

The data presented indicate that DMN has a strong effect on genetic material. DNA alkylation and DNA repair induced by alkylation with DMN can be detected at doses where there is little or no cytotoxicity. In contrast, VDC has very little potential to damage genetic material, but can cause extensive cytotoxicity with concomitant cell regeneration and DNA replication. Thus, although VDC may have little propensity to "initiate" a tumor, it could easily serve to "promote" tumors when exposure is sufficient to cause significant tissue damage. In this respect it may resemble agents such as chloroform. These materials induce tumors in animals even though they apparently have little or no genotoxic activity, provided that they are administered at levels which cause chronic tissue damage.

Most long-term carcinogenesis bioassays do not distinguish between agents which induce tumors through direct effects on DNA and agents which enhance or "promote" a low level of spontaneous tumor formation. Although the endpoint of each process is the same (tumor formation), there are fundamental differences in the safety precautions appropriate to each case. Agents which damage DNA (genotoxic) often produce a hidden effect which is not apparent until tumors appear. In contrast, agents which produce their effects through epigenetic mechanisms (cytotoxic) should not produce tumors at levels where tissue damage is absent. Furthermore, although it is theoretically possible that a single "hit" on genetic material might be sufficient to initiate a tumor (if one ignores the role of DNA repair mechanisms), it is clear that multiple "hits" in the cytoplasm would be required to kill a cell. Consequently there is ample precedent for suggesting that the rela-

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be lost much more to investigate this point. [^{14}C]VDC exposed mice after exposure showed radioactivity was half-lives of less than 100 hr since it was shown that exposure to the VDC (50 ppm) did not inhibit normal DNA pre-replicative thymidine (Table 1). Radioactivity was studied (192 hr), it was found that radioactivity found in the liver to [^{14}C]VDC is due to the DNA repair process which represents direct incorporation of C-14 into the DNA during of DNA repair. This small amount of radioactivity is a significant observation. Mechanisms are thought to be in protecting organisms from the effects of amounts of genetic damage.

A direct comparison of DMN and VDC was made in an experimental procedure. DNA repair *in vivo* (as measured by DNA damage in [^3H]thymidine) was compared to DNA synthesis in mice given urea (400 mg/kg).

There was a dramatic increase in HU-resistant [^3H]thymidine incorporation (Table 3). The ratio of incorporation (Repair index) was elevated at the highest dose of VDC. In a dose-dependent manner, VDC exposure resulted in a decrease in this ratio. The ratio of mice exposed to VDC in the livers or kidneys of mice given VDC, was not

significantly elevated. Although the levels of repair observed after VDC exposure were too low to permit the evaluation of their dose dependency, they were clearly of a different order of magnitude than the DNA repair induced in the livers of DMN-injected mice.

Since the observation of DNA repair implies that some sort of previous alteration to DNA has taken place, the low potency of VDC in this system again suggests that its *in vivo* potential for producing mutagenic effects is much less than that of DMN. The failure to demonstrate significant genetic effects with tumorigenic doses of VDC suggested that epigenetic mechanisms might be operating.

The first suggestion that epigenetic (cytotoxic) factors may be important in the tumorigenicity of VDC came from Maltoni *et al.* (1977) who reported: "There is a clear-cut direct relationship between the degree of toxic (tissue damage) and carcinogenic effects in the different animal species and sexes considered."

There are several ways in which the repeated induction of tissue damage sufficient to cause regeneration could influence the rate of tumor expression. First, there will obviously be a much greater number of replications of DNA in a tissue where a significant number of cells are replaced by regeneration during each day of a two year study than in undamaged tissue. Even if such replication occurred with very high fidelity, there would be an increased chance for spontaneous errors and hence mutation. Furthermore, sites of damage in the DNA arising from background sources (e.g., cosmic radiation) may not be recognizable as "errors" after replication. This suggests that the DNA repair systems might lose some of their effectiveness in protecting the organism from the consequences of low levels of genetic damage when cells are regenerating rapidly. Berman *et al.* (1978) found that increased division of cells enhanced their susceptibility to mutagenic events. McCormick (1979) also reported

that momentarily arresting cell division to give added time for DNA repair greatly diminished the mutational activity of uv light. In this context it is noteworthy that most of the chemical and physical treatments which act as promoters of carcinogenesis also stimulate DNA synthesis (Berenblum, 1944; Marx, 1978). Although it is not clear which of these mechanisms may be significant biologically, it is clear that when the doses of chemical employed in a carcinogenesis bioassay are toxic enough to stimulate cell regeneration, epigenetic factors cannot be excluded.

In order to determine whether cell division might be important in the oncogenicity of VDC, an experimental procedure for measuring DNA synthesis was developed. This procedure differs from that employed to measure DNA repair in two important respects:

- (1) Hydroxyurea is not used to suppress DNA replication.
- (2) [^3H]TdR is injected approximately 48 hr after the event suspected of causing tissue damage.

Since normal replicative synthesis is much more extensive than the HU-resistant repair synthesis of DNA, and since repair synthesis declines rapidly in the first few hours following carcinogen exposure (R. H. Reitz, unpublished data), this procedure measures primarily DNA replication.

In contrast to its weak genetic effects, VDC produced a dramatic change in DNA synthesis in mice (Table 4). After exposure to 50 ppm VDC for 6 hr, the DNA replication in the kidneys of male mice was elevated 25-fold. This effect was also seen after exposure to 10 ppm of VDC (6 hr), where DNA synthesis was elevated eightfold over controls. However, the effect of VDC on replication of the DNA in the liver of male mice was much less; at 50 ppm VDC the liver DNA synthesis was 2.4 times control, while after 10 ppm VDC the liver DNA synthesis was only 1.2 times control. These

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tive tumorigenicity of cytotoxic agents should show an abrupt decrease below a critical dose.

It has been reported (McKenna *et al.*, 1977) that the capacity to metabolically activate VDC to an electrophilic species appears to be directly correlated with sensitivity to toxic effects of VDC. Thus, mice metabolize VDC more readily than rats, and are more sensitive to VDC in terms of tissue damage and attendant tumorigenicity. This association between metabolic activation and toxicity is also apparent at the molecular level. Mice consistently showed more DNA damage, and more significantly, greater elevations in the amount of DNA replication than rats exposed to equivalent doses of VDC.

For many xenobiotics, including the halogenated hydrocarbons, the rate of oxidative metabolism appears to be roughly related to the body surface area rather than the body mass (Weiss *et al.*, 1977). This implies that the metabolic activation of these materials would occur more slowly in man than in the small laboratory rodents, and indeed this appears to be true for chloroform (Reitz *et al.*, 1978), and vinyl chloride (Butcher *et al.*, 1978).

Consequently the data presented in this study support the hypothesis that the toxic effects of VDC in man, including those occurring at the molecular level and though to be associated with carcinogenicity, should be far less than those observed at equivalent dose levels in laboratory animals. Furthermore, since VDC appears to induce tumors primarily through epigenetic mechanisms, it does not appear that exposure to levels of VDC which do not cause tissue damage will pose any significant cancer risk.

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