

# Dose-response analysis of the enhancement of liver tumour formation in CF-1 mice by dieldrin

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## Abstract

The current study was undertaken to investigate the dose-response characteristics of dieldrin-mediated enhancement of liver tumour formation in CF-1 mice. The median time to tumour development was established in controls, and in dieldrin-treated animals at six levels of continuous exposure (0.1, 1, 2.5, 5, 10 and 20 p.p.m.). The results of the analysis, which was based on liver tumour data from two parallel chronic feeding studies involving 1800 mice, are at variance with those reported by Druckrey for various established chemical carcinogens. In a double-logarithmic system of coordinates there was no linear relationship between the median total dose or the median time to tumour formation and the daily dieldrin exposure level. These results suggest that the tumorigenicity of this compound in CF-1 mouse liver is determined not by the sum of all consecutive doses, but rather by the level of daily exposure, and, presumably, the duration of treatment. This concept is consistent with the observed dose-dependency and reversible nature of dieldrin-induced subcellular changes in mouse liver. These considerations, together with evidence that dieldrin and its mammalian metabolites possess neither genotoxic activity nor potential, are not inconsistent with the concept that this compound is devoid of initiating potential, and operates by enhancing the effect of a genetically linked oncogenic factor in CF-1 mouse liver.

## Introduction

Oral exposure of CF-1 mice to dieldrin results in a sustained induction of liver microsomal enzyme systems and liver enlargement associated with cellular hypertrophy and increases in total liver DNA (1-3). These effects have been found to be reversible upon cessation of dieldrin treatment (1,2).

Prolonged exposure of CF-1 mice to dieldrin may result in an increase in the incidence of liver tumours, some of which possess invasive properties and can be transplanted (4,5). Such malignant tumours may also occur in control animals. In contrast to mouse liver, dieldrin has failed to exert tumourigenic effects in livers of other experimental species (6-12). There is strong evidence that the tumourigenic action of dieldrin in mouse liver is not mediated through direct interaction of the compound or of one of its metabolites with cellular DNA. Studies of dieldrin metabolism in rats and mice (13) did not reveal a qualitative feature, i.e. a unique dieldrin metabolite, that could explain the compound's tumourigenicity in mouse liver. Furthermore, dieldrin and its metabolites gave entirely negative results when evaluated for mutagenic

activity in a variety of test systems, including the coupled liver-microsome-bacterial test system employing the liver microsomes from rats and mice (14,15). Studies have also been conducted on the binding of radioactivity to the liver cell DNA of rats and mice after exposure to [<sup>14</sup>C]dieldrin *in vivo* (2). Although very small amounts of an unidentified biotransformation product were associated with DNA there was no correlation between the extent of binding and susceptibility to tumour formation. It has also been demonstrated that acute exposure to very high doses of dieldrin does not cause DNA strand-breakage in the livers of rats and mice (2).

Other microsomal enzyme inducers, such as DDT, lindane, and phenobarbitone have also been found to enhance the incidence of liver tumours in CF-1 mice (5). It is well established that phenobarbitone and DDT can enhance the carcinogenic response of rat liver to 2-acetylaminofluorene (16-18). By analogy, it has been suggested that microsomal enzyme inducers could operate in certain strains of mouse by enhancing the effect of a pre-existing oncogenic factor, which might be genetically linked (2,3,16-18). An important implication of this postulated mechanism of action would seem to be that compounds such as dieldrin might be entirely devoid of initiating potential, and could act as enhancers of carcinogenesis rather than as carcinogens *per se*. Unfortunately, no critical experiment to distinguish between these two possible mechanisms of action has thus far been reported.

There is evidence to suggest, however, that, possibly as a general feature, enhancers of carcinogenesis and intrinsically carcinogenic agents exhibit different dose-response characteristics (19,20). It would seem to be of considerable interest, therefore, to investigate the dose-response relationship exhibited by the above-mentioned microsomal enzyme inducers in CF-1 mice to see whether or not their effects correspond to those reported for a variety of established chemical carcinogens.

Quantitative relationships in chemical carcinogenesis have been determined by calculating the total dose and time up to tumour development in 50% of the treated animals at each dose level (21-24). The plotting of these results, obtained with various carcinogens of different organotropy in a double logarithmic system of coordinates invariably demonstrated a linear relationship between the median total dose or the median tumour induction period and the daily dose level, as reflected in the equation, established by Druckrey: (daily dose) x (median time to tumour appearance)<sup>n</sup> = constant, with n > 1 (21,22) (Figure 1).

The current investigation was undertaken to establish the dose: effect relationship for dieldrin, using liver tumour data from two parallel chronic feeding studies involving a total of nearly 1800 CF-1 mice. These animal studies were carried out in the early 70s at Shell Toxicology Laboratory in Sittingbourne, UK.

## Experimental

### *Animal experiments and liver pathology*

A detailed description of experimental design and execution has been published previously (4,5), but aspects particularly relevant to the present

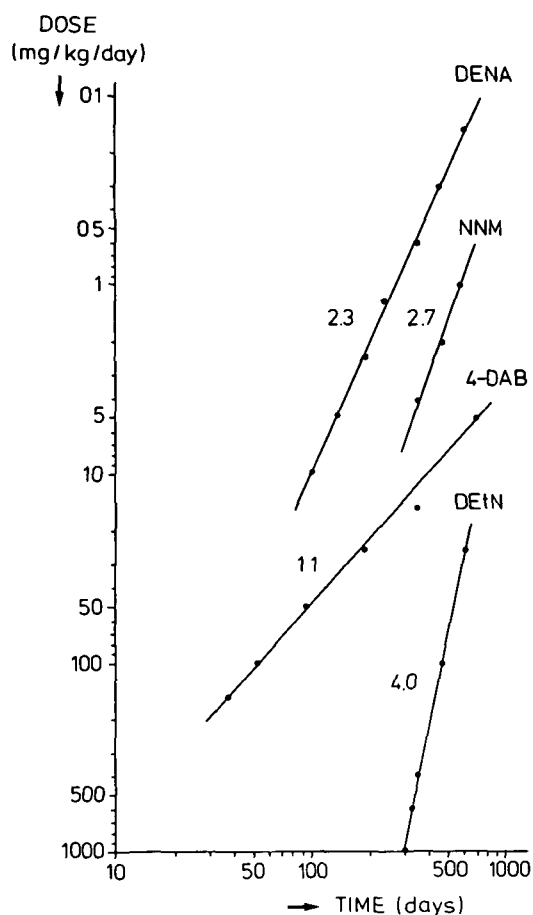


Fig. 1. "Dose-time to tumour" relationships of chemical carcinogenesis in liver. Median tumour induction periods in rats continuously exposed to the indicated dose levels of diethylnitrosamine (DENA) (21), 4-dimethylaminoazobenzene (4-DAB) (21), N-nitrosomorpholine (NNM) (24), and diethanolnitrosamine (DEtN) (22,24). Numbers indicate tangents of the slope (Druckrey's  $n$ ).

analysis are briefly reviewed. Mice of Carworth Farm No. 1 (CF-1) strain were used, bred and maintained under specific pathogen-free conditions. The animals were subjected to oral dieldrin treatment (purity of the compound > 99.9%) at the age of 3-weeks. Dose levels (p.p.m. in diet) and the numbers of animals per treatment group, divided according to sex, are indicated in Table I.

The animals were multiply housed initially, but separated into individual cages after 6-weeks of exposure. Health and behaviour of all animals were observed daily. Weekly abdominal palpation of the animals was started after 16 weeks of treatment to detect the presence of intra-abdominal masses. When such a mass was detected, the animal was palpated twice weekly and it was killed when the enlargement was considered to be detrimental to health. This procedure was adopted to ensure that as many mice as possible were available for autopsy. The treatment period up to this point will be referred to hereafter as the liver tumour induction period. A gross dissection of each mouse was made and tissue sections of a variety of organs, including liver, and any macroscopic lesions, were prepared and stained with haematoxylin and eosin. Liver tumours were classified according to the method of Walker, *et al.* (4) as adenomas (nodular growths of solid cords of parenchymal cells) and carcinomas (papilliform and adenoid growth with cells proliferating in confluent sheets with necrosis, increased mitoses, and sometimes associated metastases to the lungs).

#### Statistics

The age-specific liver tumour incidence in each treatment group and in control mice was calculated with Kaplan-Meier non-parametric estimates of liver tumour probability based on censored survival data (25), censorship being imposed on death due to other causes, natural or artificial. The fit of the tumour incidence data to a log-normal distribution was subsequently assessed. Confidence intervals for the median tumour induction period were determined as described by Brookmeyer and Crowley (26). Statistical significance of results

obtained in each treatment group relative to control data was assessed using the Logrank test, as described by Kalbfleisch and Prentice (27).

#### Results

Age-specific liver tumour incidence observed in treatment groups and in controls is shown in Figure 2. No significant differences were observed in age-specific liver tumour incidence between male and female control CF-1 mice (experiment no. 1:  $p > 0.6$ ; experiment no. 2:  $p > 0.1$ ), neither were there any significant differences between control data from the two studies for both sexes (males:  $p > 0.2$ ; females:  $p > 0.6$ ) (Table I). There was no apparent sex difference in the tumorigenic response of liver to dieldrin.

Most of the liver tumours observed in dieldrin-treated mice were diagnosed as liver adenomas, the only exception being the 10 p.p.m. dieldrin groups from the larger study, where nearly two-thirds of the tumours, both in males and females, were classified as liver carcinomas (Table I). This explains the apparent difference observed between the age-specific tumour incidence in female CF-1 mice in the two parallel studies at the 10 p.p.m. dieldrin level (Figure 2), which would appear to result from non-uniformity of the tumour type upon sacrifice. The 10 p.p.m. line (Study 2) on the left in Figure 2 consists largely of liver adenoma-bearing mice, whereas the 10 p.p.m. line (Study 1) on the right predominantly reflects the age-specific incidence of liver carcinomas.

Results generally showed a good fit to a log-normal distribution (Figure 2), and liver tumour incidence lines were more or less parallel. This evidence suggests that any fixed incidence of liver tumours may serve as an indicator of "dose: time to tumour" characteristics. This procedure was adopted in the present analysis of the "dose: time to liver carcinoma" relationship (see below).

#### Dose: time to liver tumour (adenoma) relationship

Experiments with female CF-1 mice appeared most suitable for an assessment of the dose: time to tumour relationship for two reasons: firstly, because six of the dieldrin exposure levels, i.e. 0.1, 1, 2.5, 5, 10 (study 2) and 20 p.p.m. (indicated in italics in Table I), were found to be associated with a significant reduction of the time to tumour development, as compared to controls, and, secondly, because the carcinogenic endpoint was found to be uniform across these treatment groups, i.e., most of the tumours observed upon sacrifice were classified as liver adenomas (Table I). It should be noted that a substantial proportion of both male and female mice exposed to 20 p.p.m. dieldrin died from acute intoxication within the first 13 weeks of treatment (Table I). However, since none of these early losses were due to overt hepatotoxicity, censorship was imposed on these animals, as with any animal with a cause of death other than a liver tumour. The following features were noted with respect to the median time to liver tumour development (Table I, Figure 3). There was only a minor difference of approximately two weeks in the median time to tumour development between the 20 p.p.m. and 10 p.p.m. treatments. However, a major difference of >40 weeks (~10 months) was observed between the 5 and 10 p.p.m. treatment groups. Dieldrin treatment was increasingly ineffective at dose levels < 5 p.p.m. and the age-specific liver tumour incidence in male CF-1 mice continuously exposed to 0.1 p.p.m. dieldrin was virtually identical to that seen in control animals. However, the effects seen in the female counterparts at this dose level were significant ( $p < 0.03$ ). The data were also assessed with respect to possible

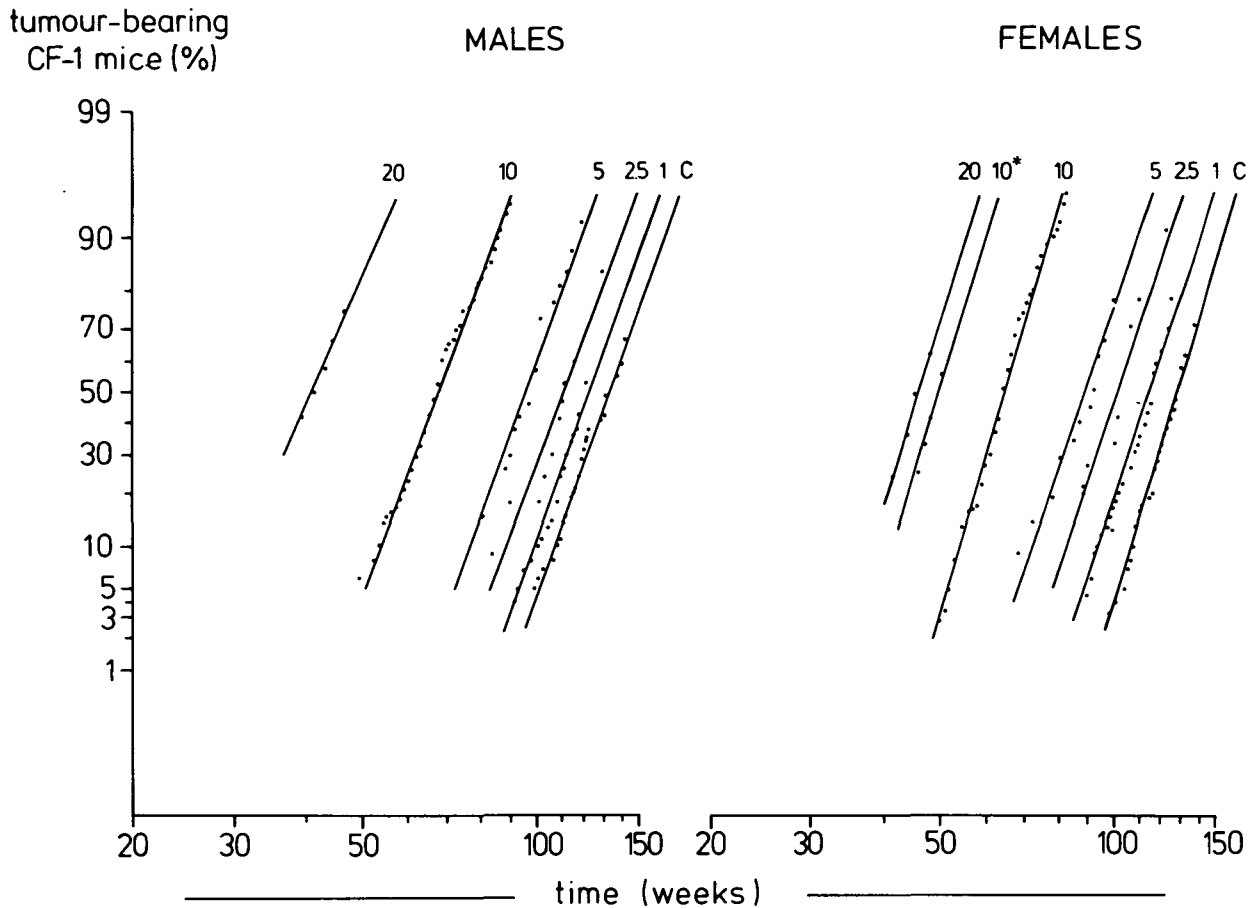


Fig. 2. Age-specific liver tumour incidence in CF-1 mice continuously exposed to the indicated daily dose levels (p.p.m.) of dieldrin. Abscissa: logarithmic co-ordinates; ordinate: Gauss distribution. For experimental details see text and Table I. "C" indicates age-specific liver tumour incidence in control CF-1 mice (study 1). 10\* p.p.m., data from experiment no. 2.

Table I. Treatment groups, liver tumour incidence, median tumour induction periods, and median tumourigenic total dose.

Sex	Experiment No.	Dietary dieldrin (mg/kg diet)	Initial number of mice	Observed number of liver-tumour bearing mice	Observed number of liver-carcinoma bearing mice	Median liver tumour induction period (weeks): T <sub>50</sub>	Median total dose <sup>d</sup> (mg/kg bodyweight): D <sub>50</sub>
Female	1	0	297	37	0	129 (> 123) <sup>b</sup>	0
	2	0	78	8	0	127 (> 123)	0
	1	0.1	120	25	4 (16) <sup>a</sup>	119 (> 117) <sup>c</sup>	8.3
	1	1	117	33	6 (18)	116 (113–120)	81.2
	2	2.5	28	13	2 (15)	106 ( 89–108)	185.5
	2	5	30	18	5 (28)	92 ( 85– 99)	322.0
	1	10	148	142	88 (62)	64 ( 64– 66)	448.0
	2	10	17	9	2 (22)	50 (> 47)	350.0
	2	20 <sup>e</sup>	21	8	3 (38)	48 (> 41)	672.0
	Male	1	0	289	58	11 (19)	133 (> 130)
2		0	78	9	0	> 128	0
1		0.1	124	33	6 (18)	130 (121–134)	9.1
1		1	111	35	10 (29)	118 (116–122) <sup>c</sup>	82.6
2		2.5	30	14	2 (14)	111 (> 109)	194.3
2		5	30	26	3 (12)	99 ( 91–101)	346.5
1		10	176	167	102 (61)	67 ( 65– 68)	469.0
2		20 <sup>e</sup>	17	12	9 (75)	41 ( 39– 46)	574.0

<sup>a</sup>Numbers in parentheses indicate incidence of carcinoma-bearing mice as a percentage of number of tumour-bearing mice. <sup>b</sup>Numbers in parentheses indicate 95% confidence intervals. <sup>c</sup>Lowest dieldrin exposure level associated with a significant reduction of median liver tumour induction period,  $p < 0.05$ . <sup>d</sup>Calculated on the basis of an average daily food intake of 100 g/kg body weight. <sup>e</sup>In the 20 p.p.m. dieldrin treatment group 11/21 female and 5/17 males died from acute intoxication within the first 13 weeks of treatment. Data in italics were used for analysis of the dose: time to tumour relationship, for reasons outlined in the text. Tumour data observed in following treatments from study 2 were considered too scant to warrant analysis: 1.25 p.p.m. (males + females), and 10 p.p.m. (males).

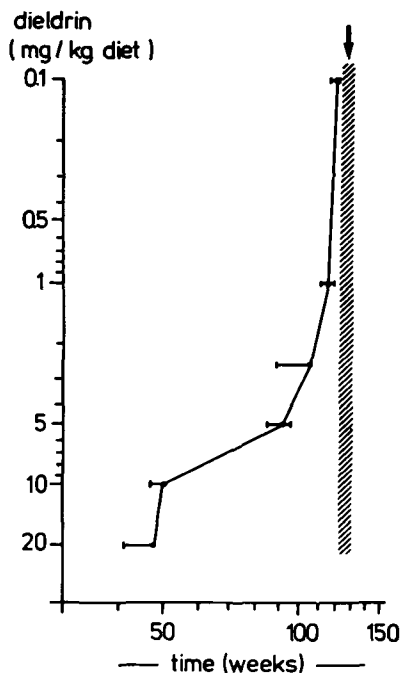


Fig. 3. "Dose: median time to liver tumour" relationship in female CF-1 mice exposed to dieldrin. Arrow indicates location of the median time to liver tumour appearance in control animals. 95% confidence intervals for treated (bars) and control animals (cross-hatched area) are also shown.

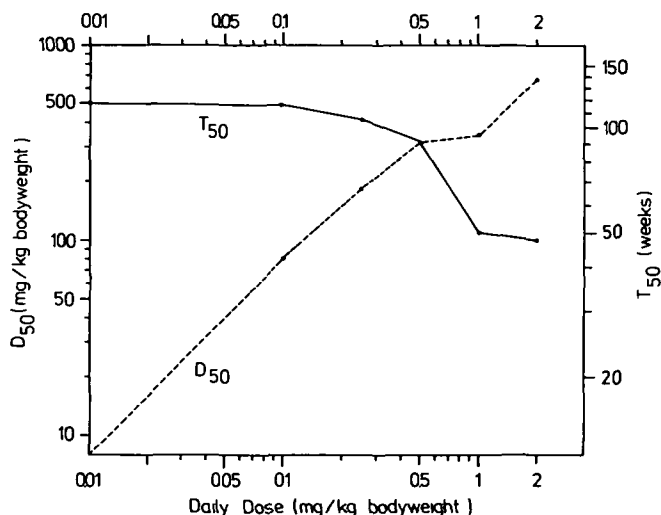


Fig. 4. Tumourigenic total dose ( $D_{50}$ ) and median liver tumour induction period ( $T_{50}$ ), as a function of the daily dieldrin dose level in chronic feeding studies with female CF-1 mice. Left ordinate: sum of all dieldrin doses administered up to 50% liver tumour appearance; right ordinate: median liver tumour induction period.

relationships between the median tumourigenic total dose ( $D_{50}$ ) and the median time to tumour development ( $T_{50}$ ) (Figure 4). The 27-week difference in  $T_{50}$  between the 0.1 p.p.m. and 5 p.p.m. dose levels was found to be associated with a near 40-fold difference in  $D_{50}$ . However, the pronounced 42-week difference in  $T_{50}$  between the 5 and 10 p.p.m. treatment groups was basically a result of a different spreading of approximately the same total dose.

#### Dose: time to liver carcinoma relationship

Median liver carcinoma induction periods could not be determined in low dose groups including control animals. In

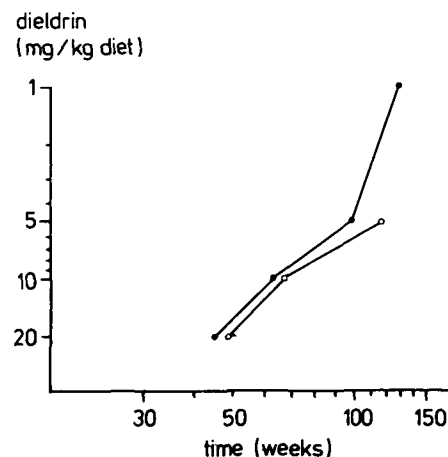


Fig. 5. "Dose-time to liver carcinoma" relationship in female CF-1 mice exposed to dieldrin. ●, 25% incidence of liver carcinomas. 1 p.p.m.: 129 weeks (>117); 5 p.p.m.: 99 weeks (>91); 10 p.p.m.: 63 weeks (62–64); 20 p.p.m.: 45 weeks. ○, 50% incidence of liver carcinomas (median induction period). 5 p.p.m.: 121 weeks (>99); 10 p.p.m. 67 weeks (66–68); 20 p.p.m.: 48 weeks (>45). Numbers in parentheses indicate 95% confidence intervals.

female mice exposed to 5 p.p.m. dieldrin, the median time to carcinoma development ( $T_{50}$ ) was found to be 121 weeks (>99). In animals exposed to 10 p.p.m. dieldrin,  $T_{50}$  was found to be 70 weeks (68–74) for males, and 67 weeks (66–68) for females. In animals treated with 20 p.p.m.,  $T_{50}$  was 44 weeks (<48) for males, and 48 weeks (>45) for females. (Numbers in parentheses represent 95% confidence intervals.)

In view of this limited evidence, it was decided to use a 25% incidence, of liver carcinoma(s) in dieldrin-treated mice as an indicator of the "dose: time to carcinoma" relationship. The results of this analysis (Figure 5) were similar in pattern to those reported above for the induction of liver tumours (comprising mainly adenomas) (Figure 3) except that the differences between the 10 and 20 p.p.m. treatment groups were more pronounced with respect to the induction of carcinomas.

#### Discussion

A number of microsomal enzyme inducers, i.e. DDT, dieldrin, lindane and phenobarbitone, have been found to increase the incidence of liver tumours in CF-1 mice (4,5). According to current definitions (28) these compounds are to be classified as carcinogenic agents *per se*, but there are indications that these compounds could operate by enhancing the effect of pre-existing oncogenic potential (1–5,13–18). Nevertheless, no critical experiment to distinguish between carcinogenesis enhancers and intrinsically carcinogenic agents has thus far been reported. There is evidence to suggest, however, that enhancers of carcinogenesis and intrinsically carcinogenic agents could exhibit different dose-response characteristics (19,20).

Druckrey and his associates have established clear dose-effect and time relationships for a variety of carcinogenic chemicals (21,22). These investigators demonstrated that, in a double logarithmic system of coordinates, linear relationships exist between the median total dose or the median tumour induction period and the daily dose level (Figure 1). A major implication of these investigations would seem to be that the effects of chemical carcinogens are a function of the sum of all consecutive doses, and thus irreversible and cumulative in

nature. The results also suggest that the concept of a threshold level is to be rejected, the only limitation in terms of tumour formation being a finite life expectancy. The current study was undertaken to establish the dose-response relationship exhibited by the putative carcinogenesis enhancer dieldrin in CF-1 mouse liver. The results of this analysis are at variance with those reported by Druckrey (21,22).

Using logarithmic coordinates, the "dose-time to tumour" relationship fits very poorly to a straight line (Figure 3: median time to liver tumour(s), mainly adenomas; Figure 5: time to liver carcinoma(s)), and it would appear unlikely therefore, that the action of this compound in CF-1 mouse liver is governed by Druckrey's equation: (daily dose) x (median time to tumour development)<sup>n</sup> = constant. The current data also fail to demonstrate a linear relationship between the tumorigenic total dose (D<sub>50</sub>) and the daily dieldrin exposure level. Although approximately linear increases in D<sub>50</sub> were observed in the daily dose level from 0.1 to 5 p.p.m., the D<sub>50</sub> values calculated for the 5 and 10 p.p.m. treatment groups were found to be very similar (Table I, Figure 4). In this latter case, a major shift in the median time to tumour development was essentially accomplished by a different daily spreading of a similar total dose. This non-linear relationship between D<sub>50</sub> and the daily dieldrin exposure level suggests that the tumorigenicity of this compound in mouse liver is not determined by the sum of all consecutive doses, but rather by the level of daily exposure and, presumably, the duration of treatment. This concept is consistent with the observed dose-dependency and reversible nature of dieldrin-induced subcellular changes in CF-1 mouse liver (1,2).

These considerations, together with evidence that dieldrin and its metabolites possess neither genotoxic activity nor potential (2,14,15) point to a different mechanism of tumorigenic action and are not inconsistent with the concept that this compound acts as a carcinogenesis enhancer in CF-1 mouse liver. In this context, it is interesting to note the striking similarities of the dose-response relationships exhibited by dieldrin in mouse liver, and by 12-O-tetradecanoylphorbol-13-acetate in 7,12-dimethylbenz[a]anthracene-initiated mouse skin carcinogenesis (20). This concept may also explain the apparent non-tumorigenicity of dieldrin in experimental species (6-12) which appear to be less susceptible to spontaneous development of liver tumours.

The results of the current study also suggest that dieldrin may not be active in mouse liver at low levels of exposure. The age-specific liver tumour incidence in male CF-1 mice exposed to 0.1 p.p.m. dieldrin was virtually identical to that seen in control animals. However, the effects seen in the female counterparts at this dose level were significant ( $p < 0.03$ ).

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