MECHANISTIC AND QUANTITATIVE ASPECTS OF LIVER TUMOUR PROMOTION IN MICE

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Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. C.C. Oosterlee,
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des namiddags te vier uur in de aula
van de Landbouwuniversiteit te Wageningen

STELLINGEN

1. De werking van de tumor-promotor dieldrin berust op een irreversibele versnelling van de oncogenese in de levers van CF-1 muizen.
   dit proefschrift

2. Dieldrin veroorzaakt een discrepantie tussen de chronologische en biologische leeftijd van de lever; de grote gelijkenis tussen de tijdverschuiving bij de tumor-vorming en de polyploidisering duidt op een oorzakelijk verband tussen beide fenomenen.
   dit proefschrift

3. De bepaling van de hepatocellulaire polyploidiseringsgraad in muizen biedt goede perspectieven voor de ontwikkeling van een toets voor de screening van stoffen op levertumor promoverende eigenschappen.
   dit proefschrift

4. De polyploidisering van hepatocyten ten gevolge van dieldrin behandeling komt voornamelijk tot stand door DNA synthese en veel minder door kernfusie.
   dit proefschrift

5. Tumor-promotoren zijn niet in staat in cellen of organen nieuwe karakteristieken te introduceren; hun werking berust op de beinvloeding van bestaande fysiologische mechanismen.

6. Apoptosis (controlled death) van cellen in lever foci na beeindeging van de behandeling met tumor-promotoren (Bursch et al.) kan, gezien de irreversibele effecten van zowel dieldrin (dit proefschrift) als phenobarbital (Peraino et al.) op de levertumor ontwikkeling, het ogenschijnlijk verdwijnen van deze foci niet verklaren.

   W. Bursch, B. Lauer, I. Timmermann-Trosiener, G. Barthel, J. Schuppler and R. Schulte-Hermann. Carcinogenesis 5 (1984) 453-458.

   C. Peraino, R.J.M. Fry, and E. Staffeld. Cancer Res. 37 (1977) 3623-3627.

7. Bij het onderzoek naar de mutagene werking van een stof dient de S9 mix bij voorkeur te worden bereid uit een met dezelfde stof geinduceerde lever.

8. De conclusie van Silber et al. dat de genen voor L- en K-pyruvate kinase isoenzymen niet gelijktijdig actief zijn omdat er geen L-K hybride van dit enzym in hepatocyten voorkomt is voorbarig.

   D. Silber, E. Checinska, J. Rabczynski, A.A. Kasprzak and M. Kochman. Europ. J. Cancer 14 (1978) 729-739.

9. De door de EG vastgelegde maximaal toelaatbare concentratie voor bestrijdingsmiddelen in drinkwater (0.0001 mg/l) houdt onvoldoende rekening met de toxicologische heterogeniteit van deze stoffen.

10. Het bestaan van een of meerdere z.g. Super Attractor(s) (Dressler et al.) zou zowel de beweging van de melkweg t.o.v. de achtergrond straling als ook het theoretische tekort aan (waargenome) massa in het heelal kunnen verklaren.

    A. Dressler, S.M. Faber, D. Burnstein, R.L. Davies, D. Lynden-Bell, R.J. Terlevich, and G. Wegner. Astrophysical J. 313 (1987) 37-42.

11. Bij de bepaling van de "acceptable daily intake" voor lichaamsvreemde stoffen (1/100 van de "no observed effect level") wordt onvoldoende rekening gehouden met mogelijke synergetische effecten van de steeds groter wordende aantallen lichaamsvreemde stoffen; hierdoor kunnen risico's voor de gezondheid onderschat worden.

12. Astrologie oefent op vele mensen dezelfde facinerende werking uit als "roddelbladen" en heeft inhoudelijk dezelfde waarde.

Ben van Ravenzwaay
Mechanistic and Quantitative Aspects of Liver Tumour Promotion in Mice
13 mei 1988

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VOORWOORD

Dit proefschrift is tot stand gekomen dankzij een cooperatie tussen de vakgroep Toxikologie van de Landbouw Universiteit te Wageningen en de vakgroep molekulaire Toxikologie van het Deutsches Krebsforschungszentrum te Heidelberg, Bondsrepubliek Duitsland.
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INTRODUCTION

1. INTRODUCTION

1.1 General Introduction and Objectives of the Study

Some features that make cancer cells different from normal cells are:

1) Cancer cells grow and divide with less restraint than normal cells in which division is closely regulated.
2) Cancer cells are, more or less, dedifferentiated and therefore do not perform all of their normal functions.
3) Cancer cells are immortalized and therefore do not die on schedule.

The result of these cellular characteristics is an overgrowth (tumour) of misfunctioning cells that interferes with the activities of normal cells and tissues.

The eukaryotic cell cycle (Fig. 1) can be divided into 4 phases:

- G1, as the period between mitosis and the beginning of DNA syntheses,
- S, the period of DNA synthesis,
- G2, the premitotic interval,
- M, the period of mitosis.

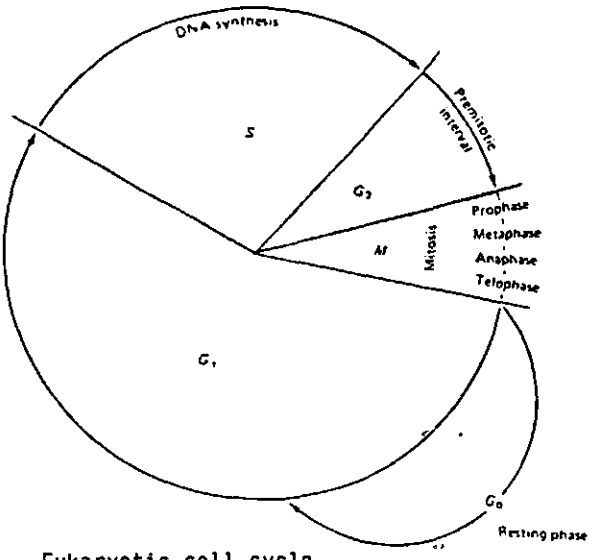


Fig. 1. Eukaryotic cell cycle.

S, G2 and M periods are relatively constant in most cells (Pardee, 1978) whereas the length of the G1 phase can vary considerably in different cells (Gross, 1968). It was thus hypothesized that tumour growth would result from a shortened G2 phase. However, it was observed that the cell cycle of cancer cells is, generally, not shorter than that of normal cells (Braun, 1974), contradicting the aforementioned theory. Since cellular division in cancer cells is not faster than in normal cells, tumour growth could occur when cells divide without needing to do so. In adult animals, cells divide only to replace other cells which were lost, in this way a tight balance between cell loss and gain is maintained. Tumour growth thus takes place because cancer cells divide, although there is no need for such a division.

Many studies have clearly demonstrated that numerous cellular constituents normally present during embryonic or fetal life, but absent in tissues or organs of mature individuals, reappear in neoplastic tissues. Among the common features of embryonic and neoplastic cells are: α-fetoprotein (Abelev, 1963), carcinoembryonic antigen (Fischman, 1976) and several isoenzymes, such as pyruvate kinase-K (Weinhouse, 1972), lactic dehydrogenase-M4 (Fischer, 1983) and aldolase-A (Schapera, 1973). The loss of tissue specific functions, with a concomitant re-expression of fetal gene products, has been found to be more pronounced in tumours which are more malignant (Wilis, 1967). Apparently, an inverse relationship exists between differentiation and cellular division (Fig. 2).

The fetal characteristics of cancer cells have initiated many investigations concerning the role of stem cells, i.e. cells which have retainted their proliferative capacities and which are not fully differentiated, in carcinogenesis.

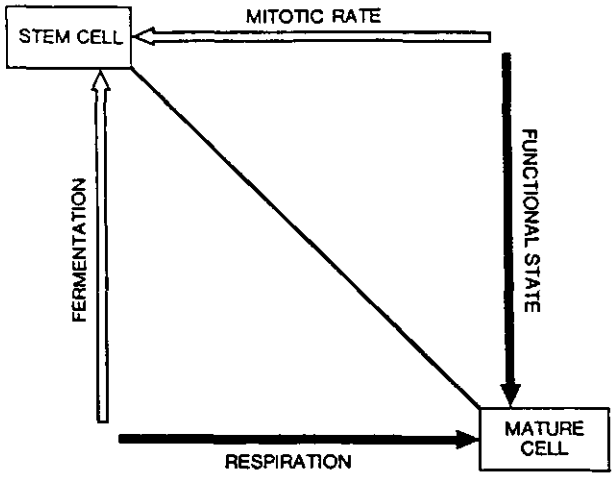


Fig. 2. Characteristics influenced by the state of cell maturation.

From such studies on testicular teratocarcinoma (Pierce, 1967), Pierce (1970) concluded that in carcinogenesis the target cell is the stem cell normally present in tissues. This concept therefore excludes adult or mature cells as possible targets for neoplastic transformation. A somewhat different concept, along the same line of reasoning, was developed by Potter (1969) and Potter et al. (1972), which was formulated as: "Oncogeny as blocked ontogeny". In their view, cancer would develop if stem cells (or other cells with proliferative capacity) are arrested in their differentiational development, and are thus forced to display a higher rate of proliferation (see Fig. 2). This hypothesis could explain the large diversity of neoplasms, ranging from "minimally deviated" to highly malignant varieties.

Both the aforementioned hypotheses infer that cancer originates from cells which were not fully differentiated. The question whether or not differentiated cells can undergo dedifferentiation and transformation re-

sulting in cancer, has been discussed with some controversy among cancer biologists. There is, however, some evidence for the reversibility of differentiation (Yamada, 1967; Burgess, 1974; Stone, 1950). Probably the best investigated model for the reversibility of differentiation is liver regeneration in rodents after partial hepatectomy. Bresnick (1971) summarized several cellular morphological alterations after partial hepatectomy which were also found in fetal hepatocytes. Most of these changes were also observed in preneoplastic nodules in rats treated with the carcinogen diethylnitrosamine (Bruni, 1973). Furthermore, a step-wise dedifferentiation of enzyme activity after partial hepatectomy has been reported (Curtin, 1983), which resulted in a similar enzymic pattern of regenerating, fetal and neoplastic liver. It would thus appear that differentiation in hepatocytes is a reversible process, which suggests that all hepatocytes could be a target for carcinogenic action. In this context, Uriel (1969) has advanced the thesis of "unbalanced retrodifferentiation" which holds that during the preneoplastic phase of liver carcinogenesis, hepatocytes retrodifferentiate, without the compensatory differentiation thereafter (Uriel, 1975).

The presented hypotheses "oncogeny as blocked ontogeny" and "unbalanced retrodifferentation" do not address the question as to whether a change in the genetic information (mutation) is involved in carcinogenesis or whether persistent alterations in the expression of the genetic information already present (i.e. an epigenetic mechanism) are sufficient to result in a neoplasia. Many investigators have proven beyond doubt the importance of mutagenic mechanisms involved in carcinogenesis. The close resemblance between tumour cells and fetal cells, however, suggests that changes in the expression of genetic information already present in the nucleus, may also play an important role.

Objectives of the Study

Many drugs, insecticides, food additives and other chemicals are known to induce characteristic changes in the livers of laboratory animals. These changes include liver enlargement, induction of microsomal enzyme sytems and proliferation of the smooth endoplasmic reticulum (Goldberg, 1966; Kunz et al., 1966; Wright et al., 1972). These changes are not accompanied by evidence of liver damage and are reversible upon withdrawal and elimination of the compound (Goldberg, 1966; Wright et al., 1972). Consequently, these changes are likely to be adaptive responses of the liver to increased functional demands. However, prolonged exposure of various strains of mice to xenobiotic compounds has been shown to result in increased frequencies of liver tumours in these mice (Peraino et al., 1973; Walker et al., 1973, Ruebner et al., 1981).

Several mechanisms have been proposed to explain the tumorigenic effects of microsomal enzyme inducers in mouse liver:

1) Microsomal enzyme inducers may enhance or facilitate the expression of a pre-existing oncogenic factor,
2) Microsomal enzyme inducers may be weak carcinogens themselves, and are only detected in susceptible species,
3) The induction of microsomal enzyme systems could render the liver more susceptible to tumour formation as a result of increased capability to synthesise proximate or ultimate carcinogenic forms from environmental pre-carcinogens.

The last possibility was extensively studied using dieldrin as a microsomal enzyme inducer and CF-1 mice as experimental animals (Tennekes et al., 1981). No difference in liver tumour incidence were observed between CF-1 mice bred, reared and maintained on a semi-synthetic diet and filter-paper bedding, and those exposed to a conventional diet and sawdust bedding.

Dieldrin was found to be equally tumorigenic in both environments. The dose-response characteristics for chemically induced tumour formation have been elucidated by Druckrey and co-workers (Chapter 2.2), who demonstrated that carcinogens interact irreversibly with their specific receptors, and that the result of this interaction is also irreversible, resulting in a time-dependent reinforcement factor "n". Since no evidence has been found for irreversible interactions of microsomal enzyme inducers with cellular components, it is conceivable that enhancers of carcinogenesis display different dose-response characteristics.

The first objective of this study is to establish whether dieldrin should be considered as a weak carcinogen or as a tumour promotor. To discriminate between these possibilities the dose-response characteristics of dieldrin-enhanced liver tumour formation in CF-1 mice had to be established.

The second objective of the present study was to establish qualitative and quantitative links between the functional pressure, polyploidization and liver tumour formation in CF-1 mice, using dieldrin as a model compound. Previous studies (Wright et al., 1972; Tennekes et al., 1981) have shown that the induction of liver enlargement and of microsomal enzymes is strictly dose-dependent and, most importantly, time-independent.

The enhancement of polyploidization of liver nuclei in mice exposed to microsomal enzyme inducers may not follow this pattern. Polyploidization in mice increases in the course of time (Shima and Sugahara, 1976) as well as with microsomal enzyme induction (Böhm and Noltemeyer, 1981; Schulte-Hermann, 1979). Thus, like the formation of liver tumours, polyploidization is a dose- and time-dependent process.

1.2 Dose-Response Relationships in Carcinogenesis

As the quantity of experimental results threatens to obscure the general view on the subject, the need arises to bring order into the seeming chaos. Mathematics is the only "language" that will enable us to give an exact representation of the subject.

For quantitative studies on the effects of xenobiotic compounds on carcinogenesis, knowledge is required concerning the pharmacokinetics of the applied compound.

When an (experimental) animal is exposed to a (foreign) compound, resorption is the first process to influence the ultimate effect of the compound. The second process is the distribution of the compound in the body with concomitant excretion and metabolism. Both these processes determine the dose which may ultimately reach the target cell (with its specific receptors). The reaction of the compound with specific cellular receptors is the third step, and the effect of the compound-receptor interaction is the fourth, and last, step which then may result in a biological reaction of the cell.

Druckrey and Kupfmüller (1949) gave a theoretical explanation for different dose-response relationships, based on the experiments of Clarke (1937). According to them the biological response of the cell, when exposed to a concentration C at the site of interaction, depends firstly on the reversibility of the compound-receptor interaction and secondly on the reversibility of the effect of this interaction. Three different types of dose-response relationships can be distinguished:

1. When the time constant T_R for the reversibility of receptor binding is small, i.e. the compound-receptor interaction is quickly reversible, and the time constant T_r for the reversibility of the effect is also small, i.e. the biological response caused by receptor binding is quickly re-

versible, then the biological response is proportional to the compound concentration ("Konzentrationsgift"). Therefore, the response will be completely reversible after elimination of the compound.
(Fig. 3, curve 1)

2. When either receptor binding or the effect of receptor binding is irreversible, the biological response will depend on the concentration as well as on the duration of treatment ("c·t-Gift"). In both cases the response will be additive and irreversible.
(Fig. 3, curve 2)
3. When both receptor binding and its effect are irreversible, then a time-associated acceleration of the biological response would take place ("Verstärkerwirkung"). Such a dose-response relationship would imply that the effect of a low concentration of the compound, which may not produce a significant response during the initial phases of treatment, will be amplified in time and result in an increasing response (Table I, Fig. 3, curve 3).

Table I. The theoretical basis of dose-response relationships, according to H.Druckrey and K.Küpfmüller (1949)[a]

Reversibility of receptor binding	Receptor binding in relation to compound concentration	Reversibility of the effect	Effect in relation to receptor binding	Effect in relation to compound concentration	Dose-response characteristics
$T_R \to 0$	$C_R \sim c$	$T_r \to 0$	$E \sim C_R$	$E \sim c$	Dose-dependent ("Konzentrationsgift")
$T_R \to 0$	$C_R \sim c$	$T_r \to \infty$	$E \sim \int C_R\, dt$	$E \sim \int c\, dt$	Dose- and time-dependent ("c.t.-Gift")
$T_R \to \infty$	$C_R \sim \int c\, dt$	$T_r \to 0$	$E \sim C_R$	$E \sim \int c\, dt$	Dose- and time-dependent ("c.t.-Gift")
$T_R \to \infty$	$C_R \sim \int c\, dt$	$T_r \to \infty$	$E \sim \int C_R\, dt$	$E \sim \int\int c\, dt$	Dose- and time-dependent time-associated acceleration ("Verstärkerwirkung")

[a] $T_R \to$ = time constant for the reversibility of receptor binding; $T_r \to$ = time constant for the reversibility of the effect; c = compound concentration; C_R = concentration of receptor binding; E = effect.

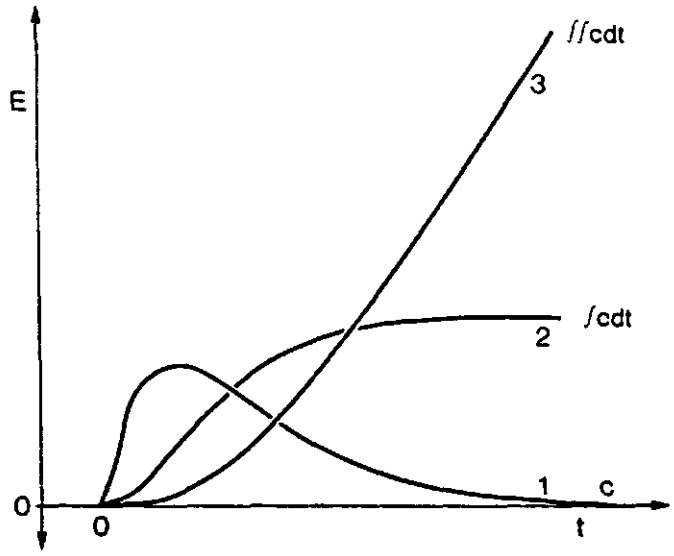


Fig. 3. Time-effect relationships for the three basic compound-receptor interactions.

In studies concerning the carcinogenic effects of 4-dimethylaminoazobenzene (4-DAB) on rat liver, Druckrey (1943) and Druckrey and Kupfmüller (1948) reported that the dose-response characteristics of this compound in the range of 10-30 mg/day can be expressed as "c·t=constant". This indicates that (liver) tumour formation is associated with a constant total tumorigenic dose. Moreover, it was concluded that this carcinogen acts irreversibly and cumulatively. At a treatment level of 1 mg/day, however, a remarkable deviation from the c·t=constant relation was observed. At this dose level (liver) tumour formation was observed after the application of a significantly lower total tumorigenic dose, when compared with the 10-30 mg/day treatment levels. In other words, the tumour induction period was shorter than expected for a "c·t=constant" relationship. In later studies (Druckrey, 1951; Druckrey and Schmähl, 1962) it was observed

that the effects of low-dose carcinogen treatment on tumour formation were accelerated in time (irreversibility of both receptor binding and its effect) and that the formulated "c·t=constant" relationship was not correct. It was merely due to the extreme low reinforcement factor of 4-DAB that an apparent "c·t=constant" relationship was observed. In a review of his own work and that of others Druckrey (1962) formulated the equation:

$$d \cdot t^n = \text{constant} \qquad (1)$$

where d = dose of the carcinogen, t = time period to 50 % tumour incidence and n (1) = a factor expressing the acceleration of carcinogenic process in time (reinforcement factor). As can be seen in Table II, "n" varies between 1.1 and 4.7 for different carcinogens and experimental animals.

Table II. Druckrey's "n" (reinforcement factor) for several carcinogens

Carcinogen	Animals	"n"	Reference
Methylcholantrene	mice	2.1	Horton and Denman (1955)
4-dimethylaminoazobenzene	rats	1.1	Druckrey et al. (1943, 1948)
3,4 benzo(a)pyrene	mice	4.0	Poel (1955)
3,4 benzo(a)pyrene	rats	4.7	Bryan and Shimkin (1943)
1,2,5,6 dibenzanthracene	rats	4.7	Bryan and Shimkin (1943)
diethylnitrosamine	rats	2.3	Druckrey et al. (1963a)
dimethylaminostilbene	rats	3.0	Druckrey et al. (1963b)
u.v. light (312 mμ)	mice	2.0	Blum (1959)
diethanolnitrosamine	rats	4.0	Druckrey et al. (1967)

In equation (1) a quantitative relationship between tumour formation and the dose level of a carcinogen is expressed. Tumour formation can be regarded as a process running at a certain velocity and should therefore be measured in units of reciprocal time. In Druckrey's words: In my reports I have clearly demonstrated, theoretically as well as experimentally,

that the latency period can be regarded as an expression of the velocity of the carcinogenic process. In the equation however, this fact is not taken into account. Rather, log reciprocal D versus log T was chosen, because the resulting equation appeared to be simple and expressive. These matters were, 25 years ago, anyhow so novel, nearly shocking. The scientific correct expression would have been:

$$(1/T)^n = k \cdot D \qquad (2)$$

which would hardly have been understood (Tennekes, personal communication with Druckrey).

The Druckrey equation not only holds for chronic exposures but was also found for single-dose experiments (Druckrey, 1967).

The fact that even a single dose of a carcinogen may lead to tumour formation emphasizes the potency of the reinforcement factor, as predicted by Druckrey and Kupfmüller (1949) in the event of irreversibility of both receptor binding and its effects.

The possibility of inducing tumours with a single dose of a carcinogen was used by Druckrey et al. (1967, 1970) to study the sensitivity of experimental animals to carcinogens in various phases of life. Some of the results obtained in these experiments are summarized in Table III.

Table III. The effects of a single dose of ethyl-nitroso-urea (20 mg/kg body weight) on rats of several ages

Age (days)	Medium Tumour Induction Period (days)	Tumour Yield (%)	Tumours/Rat
1	340	100	2.2
10	360	91	1.9
30	600	62	0.6

These results clearly demonstrate the decreasing sensitivity of rats to ethyl-nitroso-urea (ENU) with age. In a different study (Ivankovic and

Druckrey, 1968) it was demonstrated that the carcinogenic effect of ENU is highest in embryos shortly before birth. In these experiments most tumours were found in the nervus trigemini, which develops its activity a few days before birth (suckling response) and declines with increasing age. Therefore, it was suggested that during differentiation organs may be most sensitive to the effects of carcinogens.

It is essential to pay attention to the mathematical approach for dose-response relationships developed by Druckrey and Kupfmüller (1949). In their analysis the velocity of receptor binding (association) of a compound can be expressed as:

$$k \cdot C \cdot (R_0 - C_R) \qquad (3)$$

where k = reaction constant, C = concentration at the site of interaction, R_0 = total number of specific receptors (free receptor concentration at the beginning of the experiment) and C_R = number of occupied specific receptors. The velocity of dissociation can be expressed as:

$$C_R / T_R \qquad (4)$$

where T_R = a time constant indicating the extent of reversibility of receptor binding. Thus, the velocity of the changes in bound-receptor concentration (C_R) equals:

$$dC_R/dt = k \cdot C\ (R_0 - C_R) - C_R/T_R \qquad (5)$$

Druckrey and Kupfmüller (1949) assumed that the effect of receptor binding would depend upon the relative receptor binding (C_R/R_0). Substitution of C_R/R_0 in equation (5) yields

$$\frac{dC_R/R_0}{dt} = k \cdot C \cdot [1 - C_R/R_0] - \frac{C_R/R_0}{T_r} \qquad (6)$$

With compounds that bind irreversibly to receptors no dissociation of receptor binding will occur; thus, in these cases equation (6) can be modified to:

$$\frac{dC_R/R_0}{dt} = k \cdot C \cdot [1-C_R/R_0] \qquad (7)$$

In the case of chronic exposure to a constant concentration c of an irreversibly bound agent, equation (7) can be solved by integration:

$$C_R/R_0 = 1-e^{-k \cdot C \cdot t} \qquad (8)$$

Carcinogens are compounds which have been shown to bind irreversibly to their receptors. Moreover, the result of the receptor binding is also irreversible (thus, the neoplastic process is being accelerated with time). These findings are expressed in Druckrey's reinforcement factor "n". The amount of relative receptor binding, C_R/R_0, may be regarded as the proportion of the oncogenic road already covered, with cancer as the ultimate destination (when $C_R/R_0 = 1$). Thus C_R/R_0 reflects the relative carcinogenic risk (P) of an exposed individual or population. Equation (8) may thus be rewritten as:

$$P \sim 1-e^{-k \cdot C \cdot t^n} \qquad (9)$$

Carcinogenic risk can be calculated using the extended form of the Weibull model for tumour formation:

$$P = 1-e^{-[\alpha + \beta d]^m \cdot t^r} \qquad (10)$$

where: P = probability of a tumour at time t, d = dose, t = observation time, m = shape parameter for dose, r = shape parameter for the time to a tumour, α = parameter measuring the background tumour probability (P_0) with $\alpha = -\ln(1-P_0)$, β = scale parameter.

Equation (10) can be shown to be consistent with the Druckrey equation:

In Druckrey's studies the parameter α, which measures the background tumour probability, was virtually zero. For such a case, the Weibull equation can be reduced to:

$$P = 1-e^{-\beta d^m \cdot t^r} \qquad (11)$$

Considering a defined risk, e.g. P = 0.5, equation (11) becomes:

$$0.5 = 1-e^{-\beta d^m \cdot t^r} \qquad (12)$$

which can be reduced to:

$$\frac{-\ln 0.50}{\beta}^{1/m} = d \cdot t^{r/m} = d \cdot t^n = \text{constant} \qquad (13)$$

The extended form of the Weibull model for tumour risk has been shown to have an excellent fit with the experimentally observed tumour data (Carlborg, 1981). Thus, even without the knowledge of molecular events involved in carcinogenesis, Druckrey and Kupfmüller were able to provide a theoretical explanation for the dose-response relationships observed in carcinogenesis.

1.3 THE TWO STAGE MODEL

1.3.1 Introduction

One of the first theories concerning carcinogenesis was the chronic irritation theory (reviewed by Ewing, 1940). This theory was based on the observations of Pott in 1775 that chimney sweeps had a high incidence of scrotal cancer; he attributed this to their constant contact with coal tar and soot. That excessive exposure to sunlight led to the development of skin cancer, following a longer period of solar dermatitis, was also regarded as further support for the chronic irritation theory. With increasing experimental observations, however, this theory was seriously criticized and had to be abandoned.

More than half a century ago it was observed that wounding or treatment with chemical irritants accelerated the development of tumours in mouse skin, pretreated with coal tar or 3,4-benzo(a)pyrene (Deelman, 1924; Twort, 1939). A more pronounced cocarcinogenic effect on mouse skin was observed with croton oil (Berenblum, 1941), which caused a rise in tumour incidence from 0-6% in the groups receiving diluted benzo(a)pyrene alone to 37% when croton oil, or 80% when croton resin, were added. The tumour incidence with croton oil or croton resin alone was negligible. Thus, evidence was found that two distinct mechanisms were involved in carcinogenesis. This evidence was extended by the experiments of Rous and coworkers. Tar painting of rabbit ears was observed to result in the development of papillomas, when treatment was discontinued, however, the papillomas tended to regress. Renewed treatment after a long interval of non-treatment produced papillomas at the exact sites where they had previously existed (Rous, 1941). Accordingly, the concept of a tumour existing in a sub-threshold state, requiring additional aid for progressive neoplasia was formulated. The aforementioned experiments were repeated using methylcholantrene or benzo(a)pyrene as the primary stimulus, and non-carcinogenic stimuli (turpentine or mechanical injury) as the secondary treatment, with the same results (Rous, 1941; MacKenzy and Rous, 1941; Friedwald and Rous, 1944). These results were conceptualized by postulating that "carcinogenesis was composed of an initiating process, responsible for the conversion of normal into latent (or dormant) tumour cells, and a promoting process, whereby these latent tumour cells were made to develop into actual tumours" (Friedwald and Rous, 1944). The two stage model for carcinogenesis was further refined by Mottram (1944), who applied the carcinogen for the initiating action only once. A schematic summary of the experimental criteria that define the two-stage model for skin carcinogenesis is given in Figure 4.

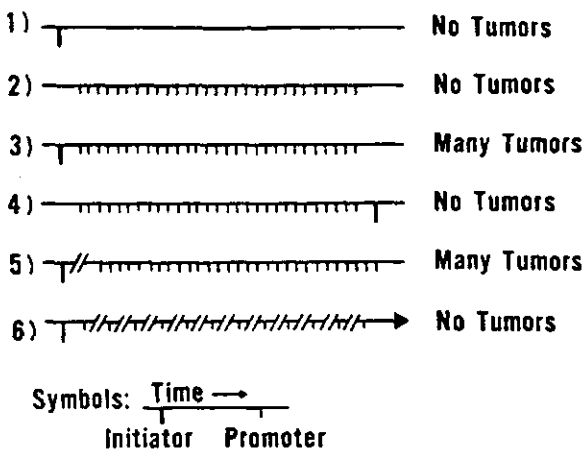


Fig. 4. Schematic summary of the experimental criteria that define skin tumour formation by the initiation and promotion components of the process of carcinogenesis.

It should be noted, however, that the two-stage model cannot always be used to explain all observations in carcinogenesis. It has been demonstrated that plant neoplasia can be established and maintained without the necessity for any alteration of the genetic information (Lutz, 1971; Binns, 1973). Further evidence for the involvement of epigenetic mechanisms in carcinogenesis was obtained in studies with virus-transformed frog renal cells. When these nuclei are transplanted into enucleated, fertilized frog eggs, some of these will develop into normal swimming-stage larvae, or tadpoles (King, 1965). Moreover, McKinnel et al. (1969) isolated triploid nuclei from the frog renal carcinoma, and conducted the same experiment as described above. However, in this study normal, triploid tadpoles developed. The importance of these studies is the demonstration that the genetic information of a cancer nucleus is sufficient for normal development of a fertilized egg cell and that non-nuclear factors play a critical role in the expression of the neoplastic potential.

1.3.2 Initiation

According to Rous' terminology, initiation may be defined as the formation of "latent" tumour cells. Since initiation can be achieved with a single application of a carcinogen (Mottram, 1944) and the thus formed latent tumour cells may be forced to express their transformation by tumour promotor treatment long after initiation (Rous and Kidd, 1941), it was concluded that initiation was an irreversible process. The fact that the neoplastic state is transferred from mother to daughter cells suggested that the irreversible change due to the initiation was to be found in the cell's genome.

Three processes are capable of inducing an irreversible change in the cell's genome and functions:

1. A change in the information in the DNA (somatic mutation theory) - resulting in the production of "abnormal" proteins.
2. A change in the control mechanism of the genome - affecting the expression of existing information in the DNA.
3. The acquisition of new information in the DNA - e.g. viral incorporation.

The somatic mutation theory for carcinogenesis was originally proposed by Boveri in 1914, long before the nature of carcinogenesis and the molecular structure of the genetic material were known. General support for the theory that initiation is caused by mutagenic events comes from the observation that most carcinogens are mutagens (McCann and Ames, 1975). Moreover, the mutation theory implies that all cells of a given tumour should be the descendants of a single cell, and that the cancer is clonal in origin. With the use of inactivated polymorphic X-linked loci in cells of women (Fialkow, 1976; Williams et al., 1983) it was shown that various tumours were indeed of clonal origin.

However, serious criticism has been raised against the mutation theory as the sole source of initiation. It has been shown that not all known carcinogens are mutagens (Rubin, 1976), and there is no good correlation between mutagenic activity of a compound and its carcinogenic action. In this context it is important to note that the impact of the mutagen, e.g. alkylation of nucleic acids (Jensen, 1978; Singer et al., 1978), is not necessarily irreversible. It was observed that the disappearance of ß-propiolacetone and methylbenzanthracene-induced adducts from DNA was too rapid to be explained by depurination or DNA turnover (Colburn and Boutwell, 1968; Rayman and Dipple, 1973). Apparently active repair of DNA can take place. Bowden et al. (1975) demonstrated excision repair of DNA lesions induced in mouse skin by ultraviolet light. Thus, when the binding level or potency of an initiator is being considered, the rate and accuracy of removal of the lesion by DNA-repair must be included in a rationalization of the effect of the initiator. The importance of DNA-repair mechanisms may be demonstrated by the disease xeroderma pigmentosum (XP). XP-patients have an impaired DNA-repair mechanism (Friedberg et al., 1979). As a result of this defect XP-patients are extremely sensitive to u.v. light induced mutations, and suffer from an incidence of skin cancer that is several thousand times higher than normal (Bridges and Strauss, 1980). Thus, the lesion induced by the initiator turns into a fixed mutation if it escapes DNA-repair (Trosko and Chang, 1978). Trosko et al. (1977) proposed that DNA-repair is involved in the initiating step of carcinogenesis, and that "error-prone" DNA-repair or replication mechanisms are the major causes of mutagenesis.

Although there is abundant evidence that mutagenesis plays an important role in initiating carcinogenesis, the other two processes capable of inducing irreversible changes in the cell's genome are also associated with neoplastic transformation. A change in the control mechanism of gene-ex-

pression, as exemplified by the translocation of the c-myc oncogene (Dalla-Fevra et al., 1983) or gene amplification (Benedict et al., 1975) has been shown to be involved in carcinogenesis.

The acquisition of new genetic information has been demonstrated to play a role in virus-associated carcinogenesis. Several viruses have been found to induce various malignancies, including sarcomas, carcinomas and hematopoietic tumours (Weiss et al., 1982). These viruses were shown to have incorporated sequences derived from cellular genes (proto-onco-genes) which gave the particular viruses the ability to transform infected normal cells (Bishop, 1983). The transforming capacity of retroviruses has been demonstrated both in vivo and in vitro (Weiss et al., 1982, Aaronson, 1983).

In conclusion, all three processes capable of inducing irreversible changes in the genome have been found to be associated with carcinogenesis and may be regarded as mechanisms for initiation.

1.3.3 Promotion

The two-stage model for carcinogenesis was based on the results of mouse skin carcinogenesis studies. Therefore, it is no surprise that most studies concerning the nature of tumour promotion were focussed on mouse skin. However, more recently, evidence was obtained that carcinogenesis is a stepwise process in many organs.

a) Skin Tumour Promotion

The introduction of croton oil as a powerful skin tumour promotor (Berenblum and Shubnik, 1947) and the isolation and identification of its tumour promotor active components, the phorbol esters (Van Duuren and Orris, 1965; Hecker and Schmidt, 1974) enabled investigators to study two-stage carcinogenesis under defined and controlled conditions. Usually mouse skin carcino-

genesis is initiated with a single dose of dimethylbenzanthracene (DMBA) followed by two applications of TPA (12-Otetradecanoylphorbol-13-acetate) (Boutwell, 1974). In a remarkable experiment in which tumour promotion was performed with two different promoting agents, it was found that tumour yield was higher when first croton oil and later turpentine was given than when first turpentine and later croton oil were (Boutwell, 1964). Accordingly, it was proposed that skin tumour promotion could be subdivided into two different stages. This concept was confirmed and elucidated further by Fürstenberger et al. (1981). Stage 1 can be induced by short term treatment with a "complete" promotor, such as TPA. Even a single application has turned out to be sufficient, provided the animal is subsequently treated by chronic application of an "incomplete" promotor (stage 2), usually a growth stimulator (Fig. 5).

Mezerein and RPA, a semi-synthetic phorbolester, are called "incomplete" promotors because, by themselves, they have only a very weak promoting capacity (Fürstenberger et al., 1981; Slaga et al., 1980). When applied, however, after a short-term exposure to TPA, they are able to promote the effects of TPA, resulting in skin tumour formation.

Skin tumour promotion has been regarded as an entirely reversible process. This concept had to be re-evaluated when Fürstenberger et al. (1983) demonstrated that the time interval between stage 1 and stage 2 promotion could be increased up to at least 8 weeks, without a significant decrease in tumour yield, implying irreversibility of stage 1 promotion.

The different characteristics of stage 1 and 2 promotion enabled investigators to discriminate between the various effects of TPA administration. Inflammation is a typical response of the skin after TPA treatment.

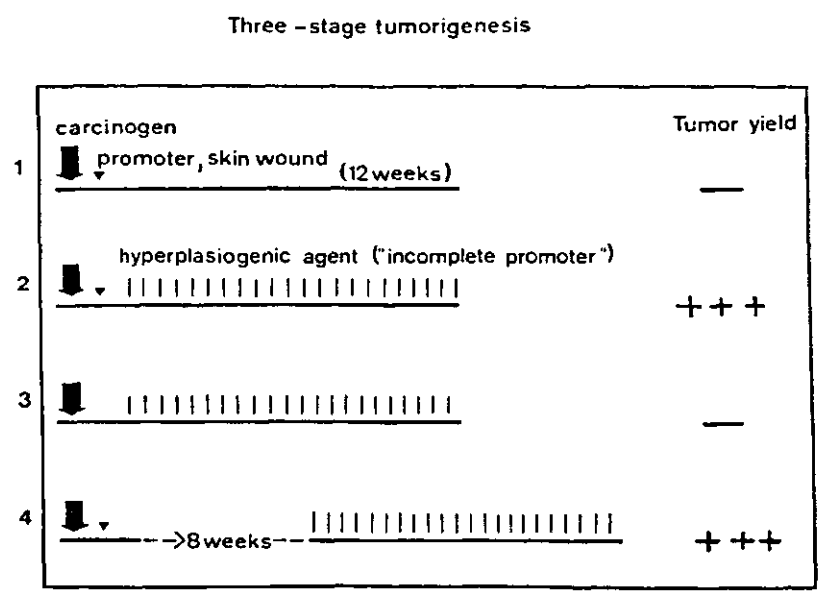


Fig. 5. A scheme of three-stage tumorigenesis in mouse skin:
1) Initiation followed by a single (or a few) local application(s) of a complete tumour promoter (or wounding) does not give rise to tumour development within a time period of 12 weeks or more;
2) a large number of tumours is obtained when initiation and limited promoter treatment is followed by chronical application of a hyperplasiogenic agent ("incomplete promoter");
3) treatment of initiated skin with an incomplete promoter alone is not tumorigenic;
4) the effect of promoter treatment in stage 1 of promotion is virtually irreversible.

However, when the promoting efficacy of different irritating agents was compared with their inflammation capacity, a relative poor correlation was found (Gschwendt and Hecker, 1974). Associated with inflammation, epidermal hyperproliferation seems to play an important role in tumour promotion. Tumour promotion was found to be completely inhibited when the TPA-induced stimulation of DNA synthesis during stage 1 was prevented by treatment of the animals with hydroxyurea (Kinzel et al., 1984). Thus, proliferation plays a role in stage 1 promotion.

Chronic treatment with incomplete promotors (stage 2) results in the induction of sustained epidermal hyperplasia (Sisskin et al., 1982) indicating that proliferation is also involved in stage 2 promotion. TPA has also been demonstrated to inhibit intercellular communication between cells (Yancey et al.,1982), an effect which has been suggested to be an important determinant of malignant transformation (Enomoto and Yamasaki, 1984). Since incomplete tumour promotors exhibit the same properties, inhibition of intercellular communication is believed to be involved in stage 2 of skin tumour promotion.

Studies on the molecular mechanism of skin tumour promotion have demonstrated the existence of a phorbol-ester receptor (Drieger and Blumberg, 1980; Ashendel et al., 1983a). Further investigations revealed the existence of a Ca^{2+}- and phospholipid-dependent, TPA-binding activity in the soluble fraction of mouse tissues, which paralleled their soluble protein kinase C activity (Ashendel et al., 1983b). The close correlation between binding affinity and promoting efficacy has led to the conclusion that receptor mediated activation of protein kinase C activity plays an important role in skin tumour promotion. Since the incomplete promotors mezerein and RPA have been found to exhibit a similar affinity to the receptor (Schmidt et al., 1983) and to stimulate protein kinase C activity almost as powerfully as TPA (Gschwendt et al., 1983), it may be concluded that these reactions are related to stage 2 promotion.

As far as the molecular mechanisms involved in stage 1 promotion are concerned, there is evidence to suggest that TPA exhibits a specific, high-affinity binding to isolated nuclei and nuclear macromolecules in mouse epidermis (Perella et al., 1982). Moreover, it has been demonstrated (Dzarlieva-Petrusevska and Fusenig, 1985) that TPA induces chromosome aberrations in mouse keratinocyte cell lines. In addition, Kinsella and

Radman (1978) have shown that TPA induces sister chromatid exchanges. They suggested that TPA treatment may lead to genetic recombination, resulting in the expression of recessive genetic changes. This hypothesis would be in agreement with the reported irreversibility of stage 1 promotion.

b) Liver Tumour Promotion

The first clear demonstration that liver carcinogenesis could also be separated into stages was reported by Peraino et al. (1973a). In this study 2-acetylaminofluorene (AAF) was fed at a concentration of 0.02% for 18 days to male albino rats to initiate carcinogenesis. Following the AAF feeding several groups of rats were exposed to 0.05% phenobarbital (a non-carcinogenic agent). After 180 days, the tumour yield in the group fed only AAF was 20%; in the groups exposed to phenobarbital as well, tumour yield was 70%, even though promotor treatment was started 30 days after the initiation, thus clearly demonstrating the validity of the two-stage model for hepatocarcinogenesis. Further studies demonstrated that compounds such as DDT (Peraino et al., 1975) or PCB's (Kimura et al., 1976) could also promote hepatocarcinogenesis. In addition, Peraino et al. (1973b) showed that the incidence of "spontaneous" liver tumour formation in male and female C3H mice (50% and 10%, respectively, after one year) could be increased to 100% for both sexes when the animals were fed on a diet containing 0.05% phenobarbital for the same period of time.

Soon after the application of an initiating agent, particularly in rat liver, groups of altered cells, or foci, can be demonstrated since they exhibit a variety of alterations (Farber and Cameron, 1980; Bannasch et al., 1985). Very useful markers to detect these foci are the altered expressions of several enzymes, such as ATPase, γ-glutamyl transpeptidase or glucose-6-phosphatase (Schwarz et al., 1984; Buchmann et al., 1985). Evidence has been presented that these enzyme-altered foci are the precursors of hepatocellu-

lar carcinomas (Watanabe and Williams, 1978; Pitot and Sirica, 1980). Tumour promotors such as phenobarbital have been shown to increase the proliferation rate of enzyme-altered foci, thus expanding the population of these altered cells (Pitot et al., 1980; Scherer and Emmelot, 1979). It has been shown that enzyme-altered foci respond more readily and more pronouncedly to phenobarbital treatment than normal hepatocytes (Schulte-Hermann et al., 1986). These responses include enhanced DNA synthesis and the expression of microsomal enzyme systems. It can thus be suggested that tumour promotors alter gene expression and thus facilitate the expression of the neoplastic potential of initiated cells (Peraino et al., 1978).

Hepatocarcinogenesis is considered to comprise three different and distinct stages: *initiation - promotion - progression* (Pitot and Sirica, 1980). Progression, the stage in which the neoplasm becomes malignant, is usually accompanied by karyotypic changes, oncogene activation and biological alterations resulting from changes in the genome (Pitot, 1986). The manifestation of a detectable tumourgenic enhancement 200 days after the cessation of a brief period of phenobarbital treatment (20 days) following AAF initiation, has led to the suggestion that phenobarbital produces irreversible changes in initiated cells (Peraino et al, 1977). Based on these and other findings, it has been put forward that liver tumour promotors, by selectively increasing the number of precursor cells, increase the risk of "spontaneous" progression at the genetic level, thus enhancing liver tumour formation (Scherer and Emmelot, 1979; Van Renselaer Potter, 1981). This view differs radically from that which is usually held for tumour promotion, especially with respect to skin carcinogenesis.

2.4 MICROSOMAL ENZYME INDUCERS

2.4.1 General Aspects

A variety of xenobiotic compounds, such as phenobarbital, DDT, α-HCH,

butylated hydroxitoluene (BHT) and dieldrin have been reported to enhance liver tumour formation in rats previously initiated with liver carcinogens (Peraino et al., 1973a, 1975, 1977; Schulte-Hermann; 1978). Chronic exposure to these compounds of various strains of mice susceptible to "spontaneous" liver tumour formation also enhances liver tumour formation (Walker et al., 1973; Thorpe and Walker, 1973; Tomatis et al., 1972; Peraino et al., 1973b; Ito et al., 1973). In terms of two-stage carcinogenesis these compounds are regarded as tumour promotors.

There is no apparent similarity in chemical structure between these compounds. Their main common features are:

1) Lipophilicity at physiologic pH,
2) Induction of liver enlargement,
3) Induction of drug-metabolising enzymes
4) Proliferation of smooth endoplasmic reticulum (SER).

The changes in the liver induced by xenobiotics are usually not accompanied by evidence of liver damage and are reversible upon withdrawal and elimination of the compound (Wright et al., 1972, 1977; Schulte-Hermann, 1974; Depiere and Ernester, 1976; Böhm and Moser, 1976).

A characteristic change in laboratory animals exposed to xenobiotic compounds is the induction of liver enlargement (Barka and Popper, 1967; Schulte-Hermann, 1974). This may be attributed to hypertrophy (an increase in cell size without an accompanying increase in DNA content), to hyperplasia (an increase in cell number or an increase in cell size with a concomitant increase in DNA content) or to a combination of the two (Barka and Popper, 1967). The process in which an increase in cell size with a proportional increase in DNA content is observed is called polyploidization and may be regarded as an arrested form of cell replication. Polyploidization is a typical feature of the liver.

The contribution of hypertrophy and hyperplasia to liver enlargement seems to depend on the properties of the inducers and on the species and strain of animal. In rats, dieldrin and phenobarbital induce predominantly hypertrophy, whereas α-HCH and BHT have been reported to induce mainly hyperplasia (Wright et al., 1972; Schulte-Hermann, 1971, 1974). In rats, dogs and rhesus monkeys, dieldrin-induced liver enlargement was mainly due to hypertrophy (Walker et al., 1978). In the mouse, however, both dieldrin and phenobarbital induced liver enlargement were reported to result from both hypertrophy and hyperplasia. Evidence has been presented that hyperplasia in mouse liver induced by xenobiotics can, at least partially, be attributed to polyploidization (Böhm and Noltemeyer, 1981; Schulte-Hermann, 1979).

Inducers of liver enlargement have been found to be substrates of the microsomal mono-oxygenase system of mammalian liver and were able to induce the activity of these enzymes (Conney, 1967; Wright et al., 1977). The mono-oxygenase or mixed-function oxidase systems catalyse the oxidative catabolism of lipophilic substrates and require NADPH and molecular oxygen for their action. This membrane bound system consists of an electron donating system (NADPH-cytochrome P-450 reductase, cytochrome b5 reductase and cytochrome b5), and iron-containing haemoproteins. On the basis of the spectral properties of the haemoproteins, this system is referred to as P-450 (Omura and Sato, 1964). The P-450 system metabolises not only endogenous substrates such as steroids or fatty acids but also a variety of lipophilic xenobiotics (Conney, 1967; Gilette et al., 1972). Its very broad substrate specificity can, at least partially, be explained by the existence of many isoenzymes. In the rat liver 11 P-450 isoenzymes have been purified, all with different activities toward a number of specific substrates (Thomas et al., 1983; Ryan, 1984). The isoenzymes which are most frequently induced by xenobiotic compounds are the b, c, d and e forms (Table IV).

Table IV. INDUCTION OF CYTOCHROME P-450 ISOENZYMES

Inducer	Total Cytochrome P-450 (nmol/mg)	Isoenzymes (% of total amount of cytochrome P-450)				
		a	b+e	c	d	unknown
none	0.86	6	4	3	5	82
isosafrole	2.33	5	17	16	38	24
3-methycholanthrene	1.83	14	1	78	24	-16
beta-naphtaflavone	1.96	9	1	71	24	-4
TCDD	2.51	13	1	60	20	7
arochlor 1254	3.67	7	37	27	22	7
gamma-chlordane	1.88	4	46	3	1	46
phenobarbital	2.18	6	55	1	2	36
SKF 525-A	2.26	4	35	4	1	56
PCN	1.34	5	3	2	1	89

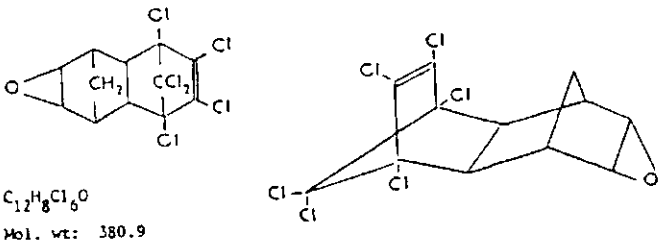
The exact nature of the relationship between microsomal enzyme induction and liver tumour promotion is not known. However, Poland and co-workers have provided evidence for a receptor-mediated, liver tumour-promoting effect of microsomal enzyme induction. The aryl hydrocarbon hydroxylase (AHH) activity is generally used to determine the induction of the P-450 c isoenzyme by polycyclic aromatic hydrocarbons, such as benzo(a)pyrene and methylcholantrene. One of the most potent liver tumour promotors, inducing the P-450 c form (Table IV), is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Van Miller et al., 1977; Pitot et al., 1980). Studying the relationship between different chlorinated dibenzo-p-dioxins and their efficacy in inducing AHH activity, Poland and Glover (1976) were able to identify a receptor for these compounds. The ability of the different chlorinated dibenzo-p-dioxins to induce AHH activity was found to be related to their

toxicity (Poland and Knutson, 1982). Interestingly, AHH activity (i.e. the P-450 c isoenzyme) cannot be induced in all mouse strains (Nebert and Gielen, 1972; Thomas et al., 1972). A mutation in the structural gene for the AH-receptor in DBA/2 mice has been shown to be responsible for the impaired responsiveness of these mice to microsomal enzyme induction by polycyclic aromatic hydrocarbons (Okey et al., 1979; Weaver et al., 1980). Moreover, it has been shown that these mice are also non-responsive to tumour promotion by these compounds. Poland et al. (1982) have also demonstrated the existence of such a receptor in mouse epidermis and the correlation of the existence of this receptor with tumour promotion. It would thus appear that liver tumour promotion by xenobiotic compounds may be mediated by receptors responsible for the induction of microsomal enzyme activity.

1.4.2 Dieldrin

Dieldrin was first synthesized in the laboratory in 1948, and commercial production in the USA was first reported in 1950 (Galley, 1970). The only known use for dieldrin is as an insecticide, in 1972 it was estimated that 80% of the USA production of dieldrin was used on corn crops and about 10% for termite control (IARCmonographs, 1974).

The chemical name of dieldrin is: endo-exo isomer of 1,2,3,4,10,10-hexachloro-6,7-epoxi-1,4,4a,5,6,7,8,8a-octahydro-1,4,5,8-dimethano naphthalene, its chemical structure is shown in Figure 6.
Dieldrin is a solid chemical with a melting point of 176-177°C. Its density is 1.75 mg/ml; it is not soluble in water, but readily dissolves in acetone, benzene or DMSO.



$C_{12}H_8Cl_6O$

Mol. wt: 380.9

Fig. 6. Chemical structure of dieldrin.

The effects of dieldrin on tumour formation have been studied in mice, rats, dogs and monkeys. In a series of experiments, reported by Walker et al. (1973), Thorpe and Walker (1973), and Tennekes (1979) CF-1 mice were exposed to 0, 0.1, 1.0 or 10.0 ppm dieldrin in the diet. It was found that dieldrin enhanced liver tumour formation in all of the treatment groups. It should be noted however, that the CF-1 mouse strain is characterized by a high incidence of "spontaneous" liver tumours. Several studies with dieldrin in the rat have been published. Fitzhugh et al. (1964) reported a non-significant increase of liver tumours in Osbourne-Mendel rats exposed to 0.5, 2 or 10 ppm dieldrin in the diet. In female CFE rats exposed to 0.1, 1.0 or 10.0 ppm dieldrin in the diet no increase in tumour formation was observed (Walker et al., 1969). Two studies with dieldrin in Osbourne-Mendel rats (National Cancer Institute, 1978a) and in Fisher rats (National Cancer Institute, 1978b) also failed to provide evidence for a carcinogenic effect of dieldrin on rat liver.

There are few and limited data on the possible carcinogenicity of dieldrin in mammalian species other than rats and mice. Walker et al. (1969) reported that a 2-year oral exposure in dogs did not result in liver tumour formation in these animals. In another study rhesus monkeys were fed on diets containing 0, 0.01, 0.1, 0.5, 1.0, 1.75 or 5.0 mg dieldrin/kg body weight for periods up to 6.5 years. No obvious alterations in general structure in the livers of these animals were observed (Wright et al., 1978).

Epidemiological observations on the state of health of a total of 826 workers involved in the handling of dieldrin in a plant of Shell Nederland Chemie N.V. at Pernis (Rotterdam) have not demonstrated any persistent adverse effects on the health of these workers (Hoogdam et al., 1965; Jager, 1970). No deaths due to liver cancer have been observed (Jager, 1970; Versteeg and Jager, 1973).

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RESULTS

CHAPTER 2.1

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QUANTITATIVE ASPECTS OF ENHANCED LIVER TUMOUR FORMATION IN CF-1 MICE BY DIELDRIN.

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SUMMARY

The dose:response characteristics of dieldrin-mediated enhancement of liver tumur formation in CF-1 mice were analysed, using existing tumour data from chronic feeding studies at six levels of continuous exposure, involving a total of more than 1,500 animals. The dose-response relationship can be expressed as

$$D_X \cdot T_X = D_0 \cdot T_0 = \text{constant} \qquad (I)$$

where T_0 = the median liver tumour induction period in control CF-1 mice, T_X = the median liver tumour induction period in dieldrin-treated mice at a dose level D_X, D_0 = the background dose equivalent for the induction of "spontaneous" liver tumours, D_X = the sum of background dose (D_0) and actual dieldrin dose (δ_X).

The relationship, which is a Druckrey equation ($D \cdot T^n$ = constant) where $n = 1$, indicates that 1) the velocity of liver tumour development is proportional to the daily dose level (D_X), 2) the total tumorigenic dose is

constant across all doses, 3) the effects of dieldrin on the neoplastic process in mouse liver are essentially irreversible and cumulative, and 4) there is no evidence for a threshold level. However, when $\delta_x \ll D_0$, the actual contribution of dieldrin to tumour formation is expected to be negligible.

INTRODUCTION

A variety of xenobiotic compounds are known to induce characteristic changes in the livers of laboratory animals. These changes include: (1) liver enlargement, usually as a result of cell enlargement, polyploidisation or cell replication, (2) induction of drug-metabolising enzymes, and (3) proliferation of the smooth endoplasmic reticulum (1-6). Such changes are not usually accompanied by evidence of liver damage, and are reversible upon withdrawal and elimination of the compound (5,6). Consequently, this phenomenon is likely to be an adaptive response of liver to increased functional demands. However, chronic exposure of various strains of mice to microsomal enzyme inducers, such as dieldrin, phenobarbitone, DDT and hexachlorocyclohexane (HCH)-stereoisomers, may cause an increase in the incidence of liver tumours (7-12). Phenobarbitone, DDT, butylated hydroxytoluene (BHT), and α-HCH have also been shown to promote the formation of rat liver tumours from lesions previously initiated by hepatocarcinogens (13-16). By analogy, it has been suggested that microsomal enzyme inducers do not exert an intrinsically carcinogenic effect on mouse liver but function by enhancing the effect of a pre-existing oncogenic factor, which may be of environmental or genetic origin (5,7).

It is conceivable, in principle, that enhancers of carcinogenesis and intrinsically carcinogenic compounds exhibit different dose-response characteristics. The dose:response characteristics of chemical carcinogens, in

single dose and chronic exposure experiments, have been elucidated by Druckrey and his associates (17-22)

$$D \cdot T^n = \text{constant} \qquad (1)$$

where D = daily dose, T = the median tumour induction period, and n = an exponent, always greater than 1.

Thus, carcinogenic response is defined as the median time period required for a constant end-point of the carcinogenic process. Equation (1) describes a quantitative relationship between the median velocity of tumour formation (or tumour-associated death) and the dose level of the carcinogen (Druckrey, personal communication). Velocity is measured in units of reciprocal time, and, accordingly, the most appropriate form of Druckrey's relationship is

$$(1/T)^n = (\text{constant}) \cdot D \qquad (2)$$

or

$$1/T = (\text{constant}) \cdot D^{1/n} \qquad (3)$$

The dose:response characteristics of putative carcinogenesis enhancers have been studied with various model compounds, particularly with phenobarbital (23-27), but no mathematical equivalent has emerged sofar. We have recently reported a non-linear relationship between the logarithm of daily dieldrin dose and the logarithm of the median liver tumour induction period in CF-1 mice (25). This non-linearity contrasted with equation (1). However, in the dieldrin study, the likelyhood is that the velocity of tumour formation was determined by multiple factors, i.e. by combination of dieldrin dose and background factors (responsible for liver tumour development in untreated control CF-1 mice). In mathematical terms

$$1/T_x \sim D_0 + \delta_x \qquad (4)$$

where T_x = median liver tumour induction period at a dieldrin dose level δ_x and D_0 = a background dose equivalent for liver tumour development in control CF-1 mice (when $\delta_x = 0$), the velocity of which will be denoted as $1/T_0$. Accordingly, dieldrin dose relates to the increase in the velocity of liver tumour formation only, i.e. to

$$(1/T_x) - (1/T_0) \qquad (5)$$

The aim of the present analysis was to elucidate a possible quantitative relationship between dose and response, as defined above.

MATERIALS AND METHODS

Details on animal experiments, liver pathology and statistical procedures have been reported previously (8,9,25), but some important aspects are briefly reviewed. Treatment of CF-1 mice with dieldrin commenced at the age of three weeks. The animals were palpated weekly as from after 16 weeks of treatment to detect the presence of intra-abdominal masses, and killed when the enlargement was considered to be detrimental to health. The treatment period up to that point was referred to as the liver tumour induction period. Liver tumours were classified as adenomas (nodular growths of solid cords of parenchymal cells) or carcinomas (papilloform and adenoid growth with cells proliferating in confluent sheets with necrosis, increased mitosis, and sometimes associated metastases to the lungs). No significant sex difference in the tumorigenic response of liver to dieldrin could be detected (25), and tumour data for males and females within groups were combined. Details of statistical procedures have been described previously (25).

RESULTS

Most of the liver tumours observed in control CF-1 mice and in treatment groups up to the level of 5 ppm dieldrin in the diet were classfied as li-

ver adenomas (Table 1). At higher dose levels, i.e. in the 10 and 20 ppm treatment groups, appproximately 2/3 of the liver tumours were classified as carcinomas (Table 1).

Table I. Liver tumour data from long-term feeding studies with dieldrin in CF-1 mice (conducted by A.I.T.Walker and E.Thorpe at Shell Toxicology Laboratory, Sittingbourne, Kent)

Dieldrin (p.p.m.)	Initial number of mice	Number of mice with liver adenomas	Number of mice with liver carcinomas	Median tumour induction period (weeks)	Median total dose[a] (mg/kg)	Carcinoma induction period (10% incidence) (weeks)
0	586	84	11	131 (128–139)[b]	0	120 (>116)
0.1	244	48	10	122 (120–133)	8.5	119 (>117)
1	228	52	16	117 (115–121)	81.9	112 (>107)
2.5	58	27	4	108 (106–115)	189	–[c]
5	60	36	8	93 (91–99)	325	–[c]
10	324	119	190	66 (65–67)	462	60 (59–62)
20	38[d]	8	12	44 (41–48)	616	39 (<44)

[a]Calculated on the basis of an average daily food intake of 100 g/kg bodyweight.
[b]Numbers in parentheses indicate 95% confidence intervals.
[c]Carcinoma data considered too scant to warrant analysis.
[d]Sixteen animals in this treatment group died from acute intoxication within the first 13 weeks of treatment.

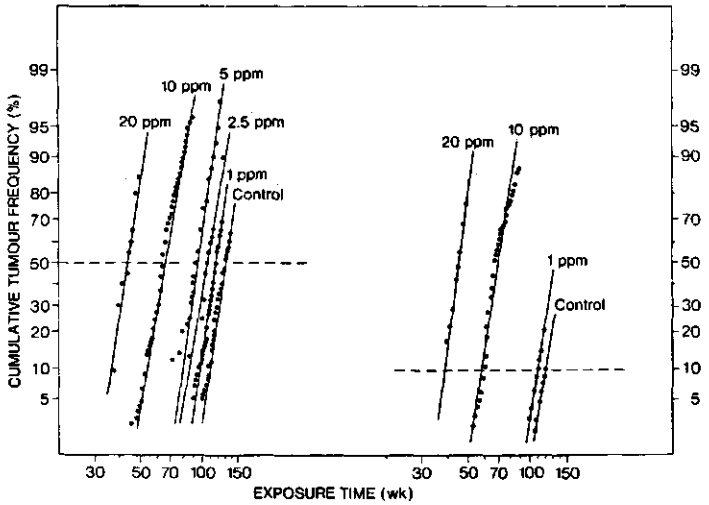

Fig. 1. Left: cumulative proportion of CF-1 mice with liver tumours versus dieldrin exposure time, at indicated dose levels (log-probit). The exposure time interval up to 50% liver tumours was designated as the median liver tumour induction period. **Right:** cumulative proportion of CF-1 mice with liver carcinomas versus dieldrin exposure time, at indicated dose levels (log-probit). The exposure time interval up to 10% liver carcinomas was designated as the liver carcinoma induction period.

A substantial proportion of the animals exposed to 20 ppm dieldrin died from acute intoxication within the first 3 months of experimentation. As with any non-liver tumour bearing animal, these early losses were

classified as incidental deaths. The cumulative incidence of liver tumours (adenomas or carcinomas) as well as the cumulative incidence of liver carcinomas (only) is shown in figure 1. Both data sets showed an excellent fit to the log-normal distribution, and linear regressions were virtually parallel. The median liver tumour induction period was defined as the treatment interval up to a 50% incidence of liver tumours. Median liver carcinoma induction periods could not be established in all but two dose groups (10 and 20 ppm, respectively). Instead, the time interval up to a 10% incidence of liver carcinomas was used as an indicator of the carcinoma induction period (Table 1). The relationship between dieldrin dose and the acceleration of tumour formation was analysed with median liver tumour induction periods as well as with liver carcinoma induction periods. Both indicators of neoplastic response yielded similar results (see below).

Acceleration of liver tumour formation and dieldrin dose

The velocity of liver tumour formation and of liver carcinoma formation versus dieldrin dose is shown in figure 2. Tumorigenic response was found to be linearly related to dose:

$$(1/T_X) = (1/T_0) + K \cdot \delta_X \qquad (6)$$

The proportionality factor of dose (K), which is the tangens of the slope, is defined as the ratio of the velocity of tumour development in control mice ($1/T_0$), and the background dose equivalent (D_0), i.e.

$$K = \frac{1/T_0}{D_0} = (1/(D_0 \cdot T_0)) \qquad (7)$$

D_0 was found to be equipotent to a level of approximately 10 ppm dieldrin in the diet (figure 2). Equations (6) and (7) lead to

$$(1/T_X) = (1/T_0) + (\delta_X / D_0 \cdot T_0)) \qquad (8)$$

or

$$(1/T_X) = (1/T_0) \cdot ((D_0 + \delta_X)/D_0) \qquad (9)$$

The sum of background dose (D_0) and dieldrin dose (δ_X) will be denoted as D_X. This modification leads to the equation

$$D_X \cdot T_X = D_0 \cdot T_0 = \text{constant} \qquad (10)$$

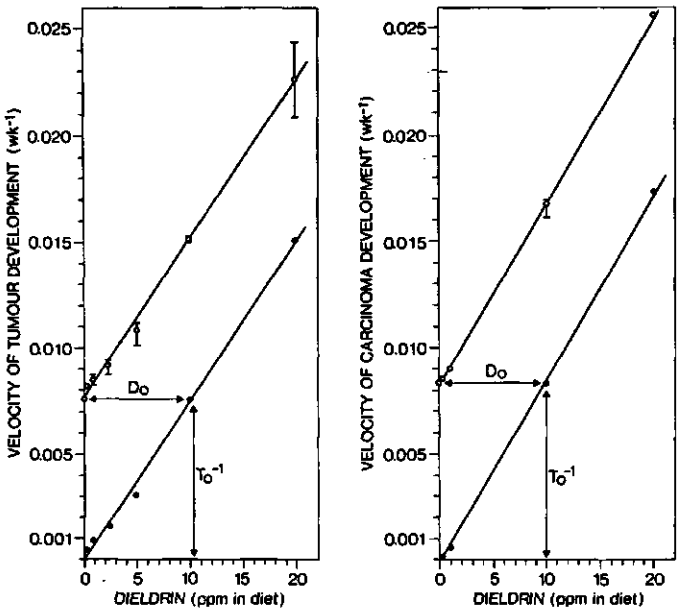

Fig. 2. Left: the reciprocal median liver tumour induction period (-○-) (= the velocity of tumour development) versus dieldrin dose. The dieldrin-associated increase in the velocity of tumour development (-●-), as defined in the text, versus dieldrin dose is also shown. The proportionality factor of dieldrin dose, which is the tangent of the angle, is defined as the ratio of the reciprocal median liver tumour induction in untreated CF-1 mice ($1/T_0$) and the background dose (D_0), which is equipotent to a level of 10.2 p.p.m. dieldrin in the diet. **Right:** the reciprocal liver carcinoma induction period (-○-) (= the velocity of carcinoma development) versus dieldrin dose. The dieldrin-associated increase in the velocity of carcinoma development (-●-) versus dieldrin dose is also shown. The proportionality factor of dose was defined as described above. The background dose (D_0) for carcinomas was found to be equipotent to a level of 10.0 p.p.m. dieldrin in the diet.

Accordingly, there is a linear relationship between the negative logarithm of the sum of background dose and actual dieldrin dose (- log D_X) and the logarithm of the median liver tumour induction period (log T_X), as is shown in figure 3. These results demonstrate that the kinetics of liver tumour formation in dieldrin-treated CF-1 mice are consistent with the Druckrey

relationship, i.e. with equation (1).

However, in the dieldrin study, the value of the exponent of time (n) equals 1, i.e. there is no time-associated acceleration of the neoplastic process in CF-1 mouse liver. The implication is that, in contrast to carcinogens ($n > 1$), the total tumorigenic dose ($= D_X \cdot T_X$) is constant across all doses. Dieldrin's actual contribution to the total tumorigenic dose is dependent on the acceleration of liver tumorigenesis, however. Equation (10) can be modified to read as

$$(D_0 + \delta_X) \cdot T_X = D_0 \cdot T_0 \qquad (11)$$

or

$$\delta_X \cdot T_X = D_0 \cdot T_0 - D_0 \cdot T_X \qquad (12)$$

or

$$\delta_X \cdot T_X = D_0 \cdot (T_0 - T_X) \qquad (13)$$

In words, the shorter the median liver tumour induction period in dieldrin-treated mice, i.e. the higher the daily dieldrin dose level, the greater will have been dieldrin's contribution to the total tumorigenic dose (table 1). This relationship is illustrated in figure 4.

DISCUSSION

The analysis yielded a simple equation to describe the dose:response characteristics of enhanced liver tumour formation in dieldrin-treated CF-1 mice. The observed quantitative relationship indicates that the total tumorigenic dose, defined is the product of the sum of daily background and dieldrin dose (D_X) and the median liver tumour induction period (T_X), is constant across all doses.

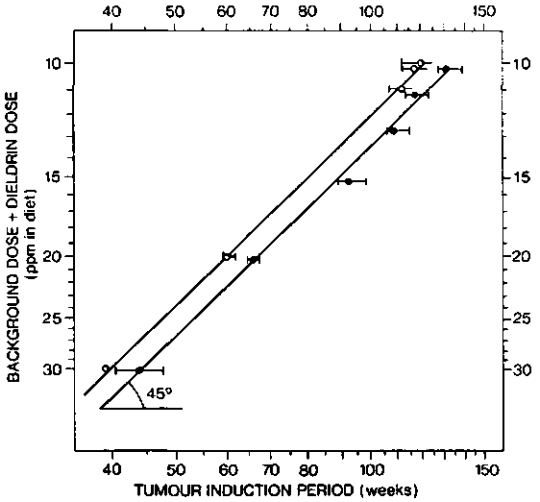

Fig. 3. The sum of background dose and dieldrin dose versus the median liver tumour induction period (-●-), and the liver carcinoma induction period (-○-), respectively, on logarithmic coordinates. The tangent of the angle (45°) is 1, in both cases. Linearity leads to equation (10).

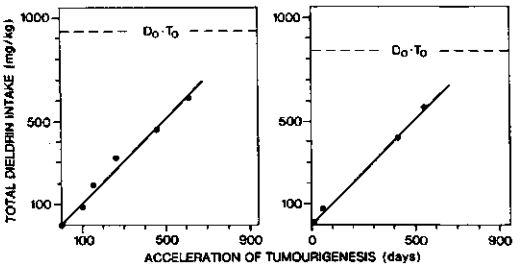

Fig. 4. Total dieldrin intake ($\delta_x \times T_x$), as a function of the acceleration of tumourigenesis ($T_0 - T_x$). **Left:** liver tumours (50%). **Right:** liver carcinomas (10%). Linearity leads to equation (13). Broken line denotes the total tumourigenic (carcinogenic) dose, which is constant, irrespective of the daily dieldrin dose level.

Table II. The theoretical basis of dose-response relationships, according to H.Druckrey and K.Küpfmüller (1949)[a]

Reversibility of receptor binding	Receptor binding in relation to compound concentration	Reversibility of the effect	Effect in relation to receptor binding	Effect in relation to compound concentration	Dose-response characteristics
$T_R - 0$	$C_R \sim c$	$T_r - 0$	$E \sim C_R$	$E \sim c$	Dose-dependent ('Konzentrationsgift')
$T_R - 0$	$C_R \sim c$	$T_r - \infty$	$E \sim \int C_R\, dt$	$E \sim \int c\, dt$	Dose- and time-dependent ('c.t.-Gift')
$T_R - \infty$	$C_R \sim \int c\, dt$	$T_r - 0$	$E \sim C_R$	$E \sim \int c\, dt$	Dose- and time-dependent ('c.t.-Gift')
$T_R - \infty$	$C_R \sim \int c\, dt$	$T_r - \infty$	$E \sim \int C_R\, dt$	$E \sim \int\int c\, dt$	Dose- and time-dependent time-associated acceleration ('Verstärkerwirkung')

[a] $T_R -$ = time constant for the reversibility of receptor binding; $T_r -$ = time constant for the reversibility of the effect; c = compound concentration; C_R = concentration of receptor binding; E = effect.

The constancy of the product of concentration and time (needed to produce a specific effect) was established for many drugs by Clarke in a remarkable monograph published half a century ago (28). A theoretical basis for "c.t. = constant" was provided by Druckrey and Küpfmüller in 1949 (18). These authors inferred that dose:response relationships are essentially determined by two processes, i.e. 1) the reversibility of binding to specific receptors in target cells, and 2) the reversibility of the effect of receptor binding (pharmacological action or toxicity). The reversibility of

any process is indicated by time constants (in this case, T_R for binding to specific receptors, and T_r for the effect of receptor binding) (table 2). When both of these time constants approach zero, i.e. when both processes are quickly reversible, the effect will be strictly dose-dependent ("Konzentrationsgift") (table 2). However, when one of two processes is irreversible, the effect will depend on dose as well as on the duration of treatment ("c.t.-Gifte") (Table 2). This has now been demonstrated to be the case for the neoplastic response of mouse liver to dieldrin (equation (10)).

Presumably, the sustained but essentially reversible interaction of dieldrin with critical receptors in CF-1 mouse hepatocytes results in irreversible progression (read: acceleration) of an ongoing neoplastic process. Finally, Druckrey and Küpfmüller predicted that the irreversibility of both processes would lead to time-associated acceleration of the effect ("Verstärkerwirkung") (Table II). Such dose-response relationships were subsequently established for chemical carcinogens of different organotropy [equation (1)] (19-22). The interaction of ultimate carcinogen(s) with critical receptors in the genome ('initiation') as well as the adverse effects of such receptor binding on the regulation of cell division and cytodifferentiation ('expression of neoplastic potential') are likely to be irreversible processes. Thus, there would appear to be a fundamental difference in the nature of receptor binding between the non-genotoxic carcinogenesis enhancer dieldrin (29-31) and liver carcinogens, such a diethylnitrosamine, where $n = 2.3$ (19), or diethanolnitrosamine, where $n = 4.7$ (22). It is interesting to note, in this context, that the dose-response relationship for dieldrin is very similar to that observed for 4-dimethylaminoazobenzene (4-DAB), where $n = 1.1$ (17,19). There is evidence to indicate that 4-DAB is a very poor initiator compared with diethylnitrosamine or diethanolnitrosamine (32).

Druckrey's equation (1) holds for single-dose as well as chronic-exposure experiments with chemical carcinogens, and identical n-values (2,3) have been ovserved in single-dose experiments with N-nitroso-N-ethylurea (21) and in chronic-exposure experiments with diethylnitrosamine (19). The 'initiation' of carcinogenesis is immediate, i.e., almost timeless in comparison with the latent period of tumors. The implication is that the second process, i.e., the expression of neoplastic potential, may well determine the kinetics of tumour development. Sharply delineated stages of functional development of hepatocytes, e.g., at birth and at the 'late suckling' period (23), have been found to be associated with high susceptibility to a single dose of a liver carcinogen (34,35). Apparently, neoplastic potential is more readily expressed when, due to drastic changes in the animals' environment, initiated hepatocytes are committed to embark on a major process of functional development. Likewise, microsomal enzyme inducers, such as dieldrin, might enhance tumourigenesis by creating a higher level of functional commitment in their target cells ('functional stress').

The results of a previous study (36) indicated that dieldrin is unlikely to exert its tumourigenic action by exacerbating the effect of a potent environmental carcinogen. No difference in liver tumour incidence was observed between CF-1 mice bred, reared and maintained on a semi-synthetic diet and filter-paper bedding, and those exposed to a conventional diet and sawdust bedding. Dieldrin was found to be equally tumourigenic in both environments (36). These experimental data suggest that the origin of background tumours may be genetically-linked and transmitted from one generation to the next. If so, this neoplastic potential is only slowly expressed, under normal circumstances. The median liver tumour induction period in untreated control CF-1 mice (T_0) is 2.5 years, which exceeds the average lifespan of the animals by ~ 6 months. The background dose (D_0) was found to be equipotent

to a level of ~ 10 p.p.m. dieldrin in the diet.

It is conceivable that D_0 reflects a certain level of naturally occurring or endogenous substances, which express intrinsic neoplastic potential in mouse hepatocytes in the course of time. Equation (10) is consistent with the view that the effects of background tumorigens and dieldrin are additive, and that there is no threshold level for the tumour-promoting action of dieldrin. However, when the actual level of dieldrin in the diet is very low in comparison with the level of background tumorigens, dieldrin's contribution to liver tumour development is expected to be negligible. Accordingly, there may be a rational for a practical 'no effect' level of carcinogenesis enhancers, such as dieldrin.

Dieldrin has been found to be non-tumourigenic in experimental species which are less susceptible to spontaneous development of liver tumours (37,38). The strain differences observed in the tumourigenic response of mouse liver to dieldrin (E. Thorpe, unpublished observations) and to phenobarbital (39) would also seem to be related to a different genetic predisposition to spontaneous tumourigenesis. Such observations are consistent with the view that pre-existing levels of tumour susceptibility in human populations could be critically important in defining virtually safe levels of tumour-promoting agents.

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CHAPTER 2.2

THE REVERSIBILITY OF SUBCELLULAR CHANGES AND ENHANCED TUMOUR FORMATION IN LIVERS OF CF-1 MICE EXPOSED TO DIELDRIN

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SUMMARY

It has recently been demonstrated that the enhancement of liver tumour formation in CF-1 mice by dieldrin is determined by the product of (tumorigenic) dose and time ("d • t = constant"). Apparently, the effects of dieldrin on the neoplastic process in CF-1 mouse liver are essentially irreversible and cumulative. This concept contrasts with the reported reversibility of dieldrin-induced subcellular changes and the absence of genotoxic activity or potential of dieldrin in mouse hepatocytes.

The results of the present study confirm that liver enlargement, microsomal enzyme induction, pyruvate kinase isoenzyme changes and nuclear polyploidization induced in mouse liver by dieldrin are

entirely reversible after withdrawal and elimination of the compound. In contrast, the tumour data obtained in a limited dieldrin exposure experiment reported by Walker et al. (Food Cosmet. Toxicol., 11, 415-431, 1972) and those obtained in a "delayed dieldrin exposure experiment", i.e. dieldrin treatment commencing after control diet feeding for a period of 60 weeks, are shown to be consistent with the concept that dieldrin exerts irreversible and cumulative effects on liver tumour development in CF-1 mice. The apparent paradox between the reversible effects of dieldrin on subcellular changes and the essentially irreversible effects on liver tumour formation can be reconsiled by assuming that any increase in the velocity of an ongoing process by whatever means of action (reversible or irreversible) for whatever period of time leads to an increased liver tumour risk.

INTRODUCTION

Continuous exposure of CF-1 mice to dieldrin results in a sustained induction of liver microsomal enzyme systems, as well as liver enlargement associated with cellular hypertrophy and increases in total liver DNA (1-3). These changes are not accompanied by evidence of liver damage, and have been reported to be entirely reversible upon withdrawal and elimination of the compound (1-3). Consequently, this phenomenon is likely to be an adaptive response of mouse liver to increased functional demands imposed by dieldrin. However, prolonged exposure of CF-1 mice to dieldrin results in enhancement of liver tumour formation. This effect can be likened to a time shift in the kinetics of "spontaneous" liver tumour

formation (4-7). A similar time shift has recently been demonstrated for the enhancement of nuclear polyploidization in CF-1 mouse liver by dieldrin (8).

The dose response characteristics of enhanced liver tumour formation in CF-1 mice by dieldrin can be expressed as (6):

$$(do + dx) \times t = \text{constant} \qquad (1)$$

where t = the median liver tumour induction period in CF-1 mice, do = the background dose (in dietary dieldrin units) for the induction of spontaneous liver tumours, and dx = the actual dieldrin dose. This empirical dose response relationship (a Druckrey relationship, where n=1) indicates that the median total tumorigenic dose is constant across all (daily) doses, and that the effects of the tumour promotor dieldrin on the neoplastic process in CF-1 mouse liver are irreversible and cumulative. This concept contrasts with the reported reversibility of dieldrin-induced subcellular changes in mouse liver (1-3). Furthermore, there is strong evidence that the tumorigenic action of dieldrin is not mediated through direct interaction of the compound or one of its metabolites with cellular DNA (2,9,10). It would appear unlikely, therefore, that the dose-response relationship reflects irreversible and cumulative binding to critical receptors in mouse hepatocytes at the genetic level. The apparent paradox can be reconciled by assuming that sustained but essentially reversible interaction of dieldrin with critical receptors in mouse hepatocytes results in irreversible progression (read : acceleration) of an ongoing neoplastic process (6).

The objective of the present study was to examine the validity of this theoretical concept. The reversibility of microsomal enzyme

induction, liver enlargement, nuclear polyploidization and pyruvate kinase isoenzyme shifts induced by dieldrin was examined 1 and 2.5 months after a 2-month treatment period with 10 ppm dieldrin in the diet. The validity of equation (1), was tested on its consistency with liver tumour data obtained in a limited dieldrin exposure experiment in CF-1 mice previously reported by Walker et al. (4). Equation (1) also implies that liver tumour formation would be associated with a constant total tumorigenic dose. To test this implication the total median tumorigenic dose was calculated for an experiment where CF-1 mice were exposed to 10 ppm dieldrin after a treatment free period of 14 months, and compared to those obtained in continuous exposure experiments at 6 dieldrin dose levels.

MATERIALS AND METHODS

Animal Experiments

CF-1 mice were kindly provided by Shell Research Ltd,Sittingbourne, Kent, UK. The colony was maintained under SPF conditions at Ivanovas, Kieslegg, FRG. Weanling female CF-1 mice were supplied to the German Cancer Research Centre upon request. The animals were allocated to groups and acclimatised for 1 week. Dieldrin treatment commenced at 4-5 weeks of age. The animals were exposed to 10 ppm dieldrin in a C-1000 diet (control and experimental diets were prepared by Altromin GmbH, Lage, FRG).Diet and water were given ad libitum.

Reversibility Experiment with Dieldrin

Approximately 20 male mice were exposed to 10 ppm dieldrin in the diet for 2 months and placed on a control diet, thereafter. Six

animals per group were killed for liver analysis 2, 3 and 4.5 months after the onset of treatment.

Delayed Dieldrin Treatment Experiment

Female animals were placed on a control diet for 60 weeks, and exposed to 10 ppm dieldrin in the diet, thereafter. The animals were palpated twice weekly as from the onset of dieldrin treatment to detect the presence of intra-abdominal masses, and killed when the enlargement was considered to be detrimental to health. The time period up to that point will be referred to as the liver tumour induction period.

Sacrifice and Preparation of Liver Tissue

Animals were weighed and killed by cervical dislocation. Livers were quickly excised, the gall-bladder was removed and the tissue was weighed. Approximately one-half of the liver was chilled in ice-cold 0.25 M Sucrose/TKM (0.05 M Tris-HCl, pH 7.4, 0.025 M KCl and 0.005 M $MgCl_2$), for the assessment of polyploidization. The remaining one-half of liver tissue was chilled in ice-cold Mic-I buffer (0.25 M Saccharose, 5.5 mM EDTA and 0.02 M Tris-HCl pH 7.5), for liver biochemistry (cytochromes P-450 and b5 concentration and pyruvate kinase isoenzyme activities). After a few minutes, the liver tissues were blotted and weighed.

Assessment of polyploidization

Liver nuclei were isolated as described by Blobel and Potter (11). Nuclear pellets were resuspended in 0.35 ml TKM buffer, and fixed by injection into tubes containing 12 ml absolute ethanol at -20° C.

Flow Cytometry

DNA analysis was performed using 4'-6-diamidino-2-phenylindole dihydrochloride (DAPI) as the quantitative DNA fluorochrome (12). Flow cytometry was carried out with a Leitz Wetzlar flow cytometer. DAPI excitation was achieved with a u.v. light source (bandpass filter 340-380 nm). DAPI fluorescence was collected through a high pass filter (430 nm). The data obtained were displayed as frequency distributions of fluorescence intensity (a measure of nuclear DNA content). In each case 40,000 nuclei were measured. The percentage of diploid and polyploid nuclei were corrected for doublets and higher aggregates of nuclei according to Beck (13).

Determination of Cytochromes Content

Liver tissue was homogenised with 4 volumes of Mic-I buffer in a Potter-Elvehjem homogeniser (10 passes, 1400 rpm). The homogenate was centrifuged at 20, 3500 and 7000xg for 10 min, respectively. The 7000xg supernatant was collected and centrifuged at 104,000xg for 1 h. The microsomal pellet was resuspended in Mic-II buffer (0.12 M KCl and 0.05 M Tris-HCl pH 7.5) and again centrifuged at 104,000xg for 1 h. The microsomal pellet was resuspended in Mic-II buffer to yield a final protein concentration (determined according to Lowry et al (14) using bovine serum albumine as a standard) of 2 mg/ml. Cytochromes b5 and P-450 concentrations were determined according to Omara and Sato (15), using a molar extinction difference of 105 and 91 $cm^{-1}mM^{-1}$ for cytochromes b5 and P-450, respectively.

Assay for Pyruvate Kinase Isoenzymes

The proportion of L- and K-pyruvate kinase (PK) was determined in the soluble fraction by measuring the pyruvate kinase activity in the presence and absence of 5 mM tryptophane, as described by Ibsen et al. (16). Tryptophane completely inhibits K-PK activity, while L-PK retained 85% of its activity (as measured by purified L-pyruvate kinase). Thus, the pyruvate kinase activity in the presence of tryptophane x 1.18 was used to calculate the L-PK activity.

Statistics

The age-specific liver tumour incidence in the delayed dieldrin treatment experiment was calculated with Kaplan-Meier non-parametric estimates of liver tumour probability based on censored data (17), 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.

RESULTS

Reversibility Experiment

A dietary dieldrin concentration of 10 ppm administered to CF-1 mice for a period of 2 months resulted in a 43% increase in relative liver weight, a 118% increase in cytochrome P-450 content and a 94% increase in cytochrome b5 content. The L-pyruvate kinase activity was decreased by approximately 15% , whereas the K-pyruvate kinase activity was 20% higher relative to untreated controls. Nuclear polyploidization, expressed in the proportion of

octaploid nuclei, had increased 88% as a result of the dieldrin treatment (Table 1).

The dieldrin-induced changes in relative liver weight, microsomal cytochrome P-450 and b5 concentrations, as well as the changes in pyruvate kinase isoenzyme activity were found to be completely reversible within one month after cessation of dieldrin treatment. The enhancement of polyploidization by dieldrin, quantitated by the proportion of octaploid nuclei, the percentage of 16c nuclei being <0.3% in all groups except in the 10 ppm treatment group at the 2-month exposure interval (1.9%), also disappeared within 2.5 months of control diet feeding (Table 1).

Table 1. The reversibility of subcellular changes in mouse liver induced by dieldrin [a]

PARAMETER	TIME - INTERVAL					
	after 2 months of dieldrin treatment		after 1 month of regression		after 2.5 months of regression	
	Control	10 ppm	Control	10 ppm	Control	10 ppm
[1]Relative liver weight	4.73 ± 0.48	6.78 ± 0.58**	4.88 ± 0.29	4.90 ± 0.41	4.29 ± 0.23	4.70 ± 0.27
[2]Cytochrome P-450	0.61 ± 0.20	1.33 ± 0.25**	0.58 ± 0.14	0.62 ± 0.08	0.80 ± 0.08	0.82 ± 0.10
[2]Cytochrome b_5	0.34 ± 0.08	0.66 ± 0.12**	0.28 ± 0.06	0.27 ± 0.03	0.42 ± 0.03	0.42 ± 0.06
[3]PK-L	45.9 ± 5.1	38.8 ± 4.0**	73.8 ± 9.8	76.7 ± 6.6	79.1 ± 7.7	78.5 ± 8.1
[3]PK-K	13.3 ± 1.7	15.9 ± 2.0*	20.2 ± 2.7	19.1 ± 3.2	21.7 ± 1.7	20.8 ± 4.3
[4]Polyploidization	9.4 ± 2.3	17.7 ± 2.4**	10.4 ± 1.9	12.5 ± 2.4*	11.6 ± 2.1	12.8 ± 1.9

a: n=6 in all cases

1: liver to bodyweight ratio (%)

2: nmol/mg microsomal protein

3: nmol pyruvate/mg soluble protein/min

4: percentage octaploid (8c) nuclei

* : $P<0.05$, ** : $P<0.01$ (significance of the difference between treatment and controls)

Limited Dieldrin Exposure Experiment

In the limited dieldrin exposure experiment in CF-1 mice reported by Walker et al. (4), the animals were exposed to 10 ppm dieldrin in the diet for periods of up to 64 weeks. The incidence of liver tumours was assessed in the survivors after 104 weeks.

The current objective is to examine whether the liver tumour data obtained in this experiment are consistent with equation (1). This examination was accomplished along the following lines of reasoning:

i) Definition of the Tumorigenic Dose.

Equation (1) implies that in a limited exposure experiment the following relation must hold:

$$dx \cdot tx = d^* \cdot t \qquad (2)$$

where dx = the (actual) dieldrin dose administered during a limited period of time

tx = the (actual and limited) dieldrin exposure time

d* = the (hypothetical) dieldrin dose level during the entire observation period (in this case 104 weeks)

t = the total observation period (104 weeks)

The tumorigenic dose level in a limited exposure experiment is consequently defined as the sum of the background dose level (do) and the hypothetical dieldrin dose level during the entire observation period (d*).

ii) Definition of the Mathematical Model.

The mathematical liver tumour risk model to be used for the prediction of the liver tumour incidence after 104 weeks in the limited dieldrin exposure experiment must be consistent with equation (1). Dose-response relationships of the form: $d \cdot t^n =$

constant (Druckrey relationships) have been shown to be a corollary of the extended Weibull model (17):

$$P = 1 - e^{-(\alpha+\beta\, d)^{m} \cdot t^{k}} \qquad (3)$$

where P = probability of a (liver) tumour at time t

d = dose

t = observation time

m = shape parameter for dose

k = shape parameter for the time to a (liver) tumour

α = parameter measuring the background (liver) tumour probability (Po) with $\alpha = -\ln(1-Po)$

β = scale parameter

The dose:response relationship for dieldrin enhanced liver tumour formation in CF-1 mice (equation (1)) is a Druckrey relation (where n=1), thus the (dieldrin) adapted form of the Weibull model for tumour incidence would be:

$$P = 1 - e^{-(\alpha+\beta(do + dx)^{m} \cdot t^{k}} \qquad (4)$$

In Druckrey's studies (19-23), the background tumour rate was essentially zero, which means that the parameter $\alpha = 0$. In the continuous dieldrin exposure study, the background factors (do) leading to "spontaneous" liver tumours in CF-1 mice were found to be equipotent to a level of approximately 10 ppm dieldrin in the diet (6). The background tumour incidence already being accounted

for by "do", a concept put forward by Albert and Altshuler (24) reduces α to 0 and leads to the following adapted form of the Weibull model:

$$P = 1 - e^{-\beta(do + dx)^{m} \cdot t^{k}} \qquad (5)$$

or, in the case of a limited dieldrin exposure experiment:

$$P = 1 - e^{-\beta(do + d^{*})^{m} \cdot t^{k}} \qquad (6)$$

iii) Test of the Mathematical Model on Consistency with Equation (1)

At a defined liver tumour risk, say P = 0.5, equation (4) can be reduced to:

$$\left(\frac{-\ln 0.5}{\beta}\right)^{1/m} = (do + dx) \cdot t^{k/m} \qquad (7)$$

Thus, equation (1) will be implied by equation (4) only if the value of the shape parameter for the time to a liver tumour (k) will be similar to the shape parameter for dose (m). This premiss was tested using the liver tumour data obtained with dieldrin in continuous exposure experiments with CF-1 mice (4,5) (fig. 1.). The tangents of the angles observed for the dose-risk relationship at 70 and 100 weeks of exposure were 8.1 and 7.5 respectively, leading to an average value of 7.8 for the shape parameter for dose (m). The tangents of the angles observed for the time-risk

relationship at the actual dieldrin dose levels of 20, 10, 5, 1 and 0 ppm in the diet were 7.3, 7.6, 7.8, 10.6 and 8.4, leading to a mean value of 7.8 $\pm$ 0.4 for the shape parameter for the time to a liver tumour (k) (the k-value for the 1 ppm data set was disregarded).

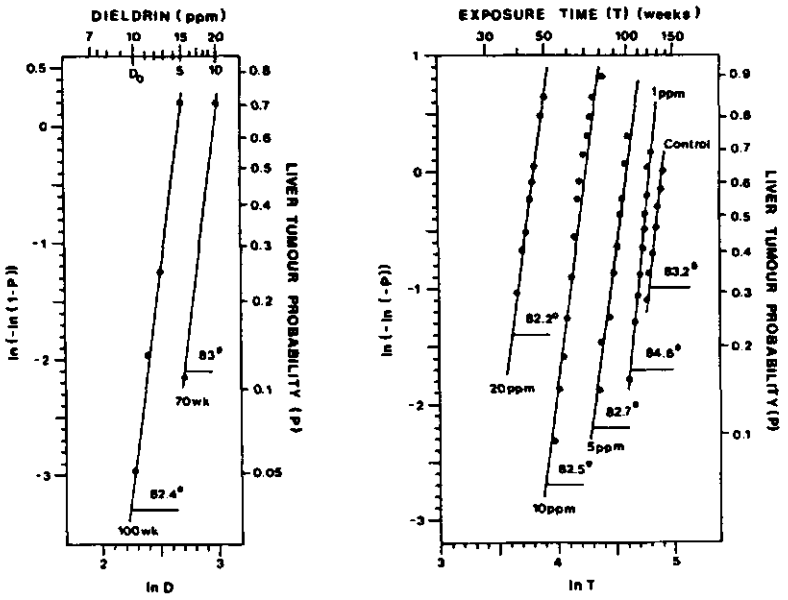

Figure 1. The fit of the liver tumour data obtained with dieldrin in continuous exposure experiments with CF-1 mice (4,5) to the extended Weibull model (males and females were combined in the absence of a sex-difference in tumour response).

P = liver tumour risk (0-1)

D = the sum of the daily background dose (do) and actual dieldrin dose (dx) in ppm in the diet

T = time in weeks

Left : Dose:tumour-risk at constant time (70 and 100 weeks).

Right : Time:tumour-risk at constant dose (0, 1, 5, 10 and 20 ppm).

Accordingly, the analysis yielded similar values for k and m, indicating that equation (1) is a corollary of equation (4). With known values of do, dx, k and m the value of β can be calculated using the median liver tumour induction periods.

iiii) Application of the Mathematical Model for the Prediction of Liver Tumour Risk in the Limited Dieldrin Exposure Study

The prediction with equation (5) of the observed incidence of liver tumours in surviving animals after 104 weeks (with exposure periods of 0, 2, 4, 8, 16, 32 and 64 weeks respectively), was accurate for 6 out of 7 groups (Table 2). Thus, the results of the limited dieldrin exposure study are consistent with equation (1) and support the concept that the effects of dieldrin on the development of mouse liver neoplasia are entirely irreversible and cumulative.

Delayed Dieldrin Exposure Experiment

Dieldrin treatment at a dose level of 10 ppm in the diet commenced after 60 weeks of control diet feeding. The cumulative proportion of CF-1 mice with palpable liver tumours is shown in Figure 2. The time interval associated with a cumulative frequency of 50% liver tumours, i.e. the median liver tumour induction period, was found to be 95 weeks (Fig. 2.).

The median liver tumour induction period in untreated control female CF-1 mice has been shown to be 129 weeks (6). Thus the administration of 10 ppm dieldrin in the diet after 60 weeks of control diet feeding accelerated the median appearance of liver tumours by 34 weeks, and effectively halved the median time to liver tumour development of 69 weeks for control CF-1 mice after 60

Table 2. Observed and expected incidence of liver tumours in male CF-1 mice fed 10 ppm dieldrin for up to 64 weeks and surving for 104 weeks

Group No.	Dieldrin Treatment Period (weeks)	Number of Survivors After 104 Weeks	Liver tumour incidence After 104 Weeks	
			Observed	Expected[x]
1	0	18	2	2.1
2	2	13	2	1.7
3	4	10	1	1.5
4	8	10	4	2.0
5	16	11	4	3.4
6	32	10	6	6.3
7	64	13	13	12.9

[x]The equation used is :

$$P = 1 - e^{-\beta (d_o + d_x)^{7.8} t^{7.8}}$$

where β = a scale parameter (3.16×10^{-25})

d_o = the backgroud dose equivalent for the induction of "spontaneous" liver tumours in CF-1 mice found to be equipotent to a level of 10 ppm dieldrin in the diet

d_x = the (hypothetical) dieldrin dose level over the entire observation interval t

t = the total observation period, in this case 104 weeks

P = the liver tumour probability,

leading to the following P-values after 104 weeks : Group 1: 0.116; group 2: 0.133; group 3: 0.152; group 4: 0.197; group 5: 0.313; group 6: 0.631; group 7: 0.994

weeks. It is interesting to note , in this context, that continuous exposure of female CF-1 mice to 10 ppm dieldrin in the diet as from the age of 4-5 weeks has been shown to result in a median liver tumour induction period of 64 weeks (Fig. 2.), which is one-half of the median liver tumour induction period in control CF-1 mice (129 weeks).

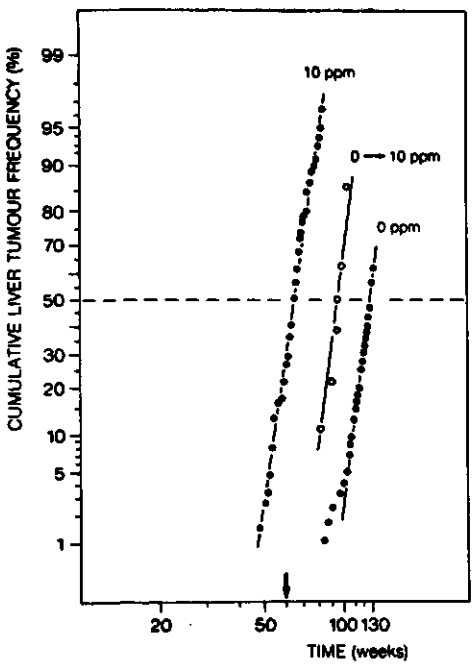

Figure 2. The cumulative proportion of female CF-1 mice with liver tumours versus time at indicated treatment levels (log-probit). The time period up to 50% liver tumours was designated as the median liver tumour induction period.

● : continuous exposure as from the age of 3 weeks (data source; references 4,5)

o : Exposure to 10 ppm after a treatment free period of 60 weeks

After 95 weeks, the total dose administered to CF-1 mice equals:

$$d_o \cdot t + d_x \cdot t_x = 10 \text{ (ppm)} \times 95 \text{ (weeks)} + 10 \text{ (ppm)} \times 35 \text{ (weeks)}$$
$$= 1300 \text{ (ppm.weeks)}$$

The corresponding total tumorigenic doses observed in the continuous exposure experiments with dieldrin in CF-1 mice were (actual daily dieldrin dose levels in parentheses) 1310 (controls), 1232 (0.1 ppm), 1287 (1 ppm), 1350 (2.5 ppm), 1395 (5 ppm), 1320 (10 ppm) and 1320 (20 ppm).

These data support the concept that liver tumour formation in CF-1 mice is determined by a defined total tumorigenic dose, regardless of the daily tumorigenic dose level and the time schedule of its administration.

DISCUSSION

The induction of subcellular changes in CF-1 mouse liver by dieldrin was found to be entirely reversible, which is in agreement with previously reported data (1,3). Relative liver weight, cytochrome P-450 and b5 concentrations as well as the isozyme composition of pyruvate kinase returned to control levels within 1 month after cessation of dieldrin treatment. These findings suggest that the reported changes in CF-1 mouse liver induced by dieldrin in the initial phases of treatment reflect an adaptation of the liver to increased functional demands. Nuclear polyploidization reached control values within 2.5 months of control diet feeding.

In contrast, the effects of dieldrin on the expression of (pre-

existing) oncogenic potential in mouse liver appears to be entirely irreversible. Three lines of evidence can be presented in support of this contention.

Firstly, the empirical dose-response relationship discovered for the enhancement of liver tumour formation by dieldrin (equation (1)) indicates that the total tumorigenic dose, which is defined as the product of the sum of daily background dose and actual dieldrin dose (do + dx) and the median tumour induction period, is constant for all dieldrin treatment levels.

Secondly, the dose-response relationship is consistent with the results of a limited exposure experiment in CF-1 mice (Table 2.). These results indicate that limited exposures to dieldrin lead to an excess liver tumour risk, the extent of which is proportional to the dieldrin exposure time interval. Similar results have been reported for the effect of limited exposures to phenobarbital of Sprague-Dawley rats pre-treated with the liver carcinogen 2-acetylaminofluorene (25). Progressive increases in the duration of phenobarbital treatment (begun 1 week after carcinogen treatment) advanced the time at which tumour enhancement occurred and similarly increased overall tumour incidence levels.

Thirdly, the enhancing effects of dieldrin on liver tumour formation in mouse liver are independent of the time of onset of dieldrin treatment. Liver tumour formation in CF-1 mice exposed to dieldrin after a treatment-free period of 60 weeks was associated with a median total tumorigenic dose virtually identical to those seen in chronic studies, with 6 levels of dieldrin exposure. The results of the present study are consistent with the concept advanced previously (6), that sustained but essentially reversible

Isolation of Liver Nuclei

Animals were weighed, and killed by cervical dislocation between 9-10 a.m. Livers were quickly excised, the gallbladder was removed, and the tissue was chilled in ice-cold 0.25 M sucrose/TKM (0.05 M Tris - HCl, pH.7,4 (20 ^{o}C), 0.025 M KCl and 0.005 M $MgCl_2$), for a few minutes. The livers were blotted and weighed. Liver nuclei were isolated as described by Blobel and Potter (16). Nuclear pellets were resuspended in 0.35 ml TKM buffer, and fixed by injection into tubes containing 12 ml absolute ethanol at -20 ^{o}C.

Flow Cytometry

DNA analysis was performed using DAPI (4'-6-diamidino-2-phenylindole-dihydrochloride) as the quantitative DNA fluorochrome (17). Flow cytometry was carried out with a Cytofluorograph 30 (Ortho Diagnostic Systems) connected to a computerized multichannel analyzer (Plurimat, Intertechnique). The UV lines (351 nm and 364 nm) of an argon ion laser were used for DAPI excitation. DAPI fluorescence was collected through a low pass filter at 450 nm. The data obtained were displayed as frequency distributions of fluorescence intensity (a measure of nuclear DNA content). In each case 40,000 nuclei were measured. The percentages of diploid and polyploid nuclei were corrected for doublets and higher aggregates of nuclei, according to Beck (18).

RESULTS

The proportion of octaploid nuclei increased linearly in all groups during the observation period of approximately one year (table 1, figure 1). The proportion of 16C nuclei was very low ($< 0.3\%$), except in the 5 and 10 ppm treatment groups at the 9-month exposure interval (0.7 %, and 1.9 % respectively). These results indicate that, with two exceptions, the losses of octaploid nuclei due to the formation of 16C nuclei were negligible.

Table I. Percentage octaploid nuclei at different treatment intervals

Dieldrin dose (p.p.m. in diet)	Treatment		Interval		
	1.85 months	3 months	6 months	9 months	14 months
0	4.1 ± 2.0 (6)[a]	4.8 ± 1.7 (11)	6.8 ± 2.3 (11)	8.5 ± 1.9 (5)	11.5 ± 2.3 (7)
0.1	–	5.5 ± 0.7 (5)	7.3 ± 1.8 (9)	9.0 ± 2.3 (5)	–
1	–	6.2 ± 1.9 (9)	7.9 ± 1.3 (8)	9.4 ± 1.1 (5)	–
5	–	8.8 ± 2.5 (10)	10.4 ± 1.7 (7)	12.5 ± 1.5 (6)[b]	–
10	12.3 ± 2.1 (6)	12.7 ± 1.6 (9)	14.5 ± 2.9 (7)	16.1 ± 2.1 (7)[c]	–

[a]Number of animals/group is indicated in parentheses.
[b]Value includes 0.7% 16C nuclei.
[c]Value includes 1.9% 16C nuclei.

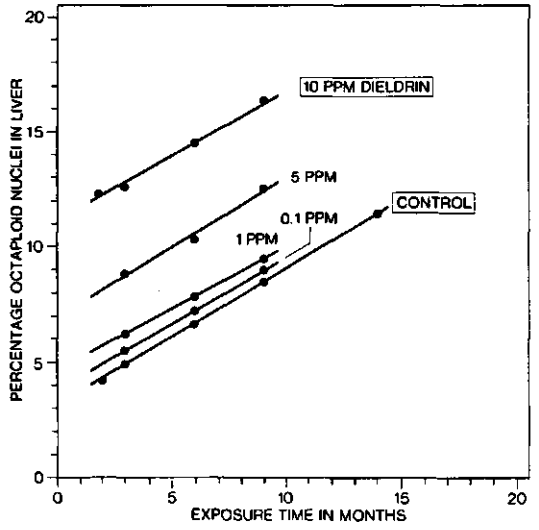

Fig. 1. Increases in octaploid liver nuclei (%) in response to dietary dieldrin concentration (p.p.m.) and exposure time.

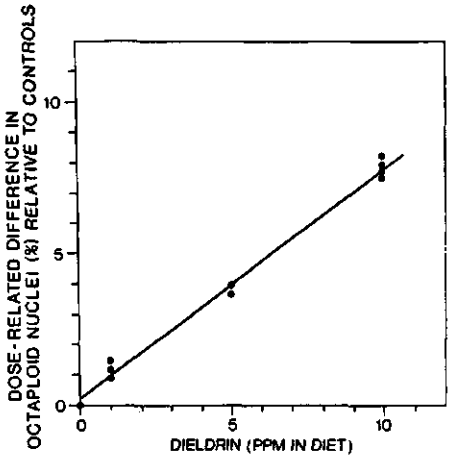

Fig. 2. Dietary dieldrin concentration-related differences (relative to controls) in octaploid liver nuclei (%) at various exposure time intervals (1.85, 3, 6 and 9 months, also see Table I).

Accordingly, the changes in the proportion of octaploid nuclei (Δ(8C)) were used as an indicator of the kinetics of polyploidisation in liver parenchyma within the period of experimental observation.

The enhancement of polyploidisation by dieldrin (measured in "steady-state" situations) was found to be proportional to dietary concentration (figure 2), and independent of the duration of dieldrin exposure. Polyploidisation was analysed in untreated control CF-1 mice and found to be proportional to time (age) (figure 3). These results show that the kinetics

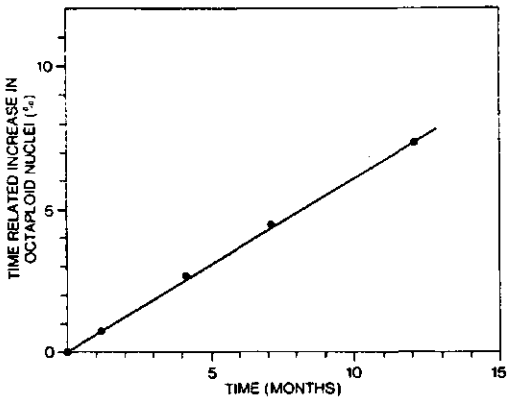

Fig. 3. Age-associated increases in octaploid liver nuclei in control mice (%). The percentage of octaploid nuclei in livers of control CF-1 mice at the 1.85 months exposure interval was used as the base line.

of polyploidisation (= the changes in the proportion of octaploid nuclei) can be expressed in ppm dieldrin in the diet (d) as well as in units of time (t) (figures 2, 3):

$$\Delta (8C) = k_1 \times d = k_2 \times t \qquad (1)$$

where k_1 and k_2 are constants.

When polyploidisation is expressed in ppm dieldrin in the diet then

$$d = (k_2/k_1) \times t \qquad (2)$$

This relationship is illustrated in figure 4. The implication of equation (2) is that the enhancement of polyploidisation by dietary dieldrin can be likened to a "time-shift" in polyploidisation associated with ageing. The extent of this time-shift which takes place in the initial phases of dieldrin treatment, i.e. before "steady-state" ist reached, is proportional to the dietary dieldrin concentration as indicated by the parallelism of the linear regressions in figure 1. Age-associated polyploidisation when expressed in dietary dieldrin concentrations can be viewed as the response of mouse liver to the (cumulative) action of a constant concentration of

toxic substances during time (t), where c which be equivalent to

d/t = 0.8 ppm dieldrin/month = 0.026 ppm/day, according to equation (2).

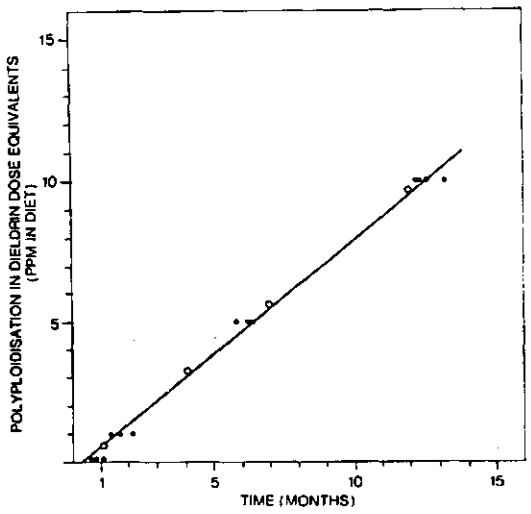

Fig. 4. Polyploidization in CF-1 mouse liver parenchyma (expressed in p.p.m. dieldrin in the diet) versus time. Age-associated increases in octaploid nuclei in control mice (○) were expressed in dietary dieldrin concentrations using the linear regression shown in Figure 2. Dieldrin-induced increases in octaploid nuclei (●) were expressed in units of time using the linear regression shown in Figure 3. Linearity leads to equation (2).

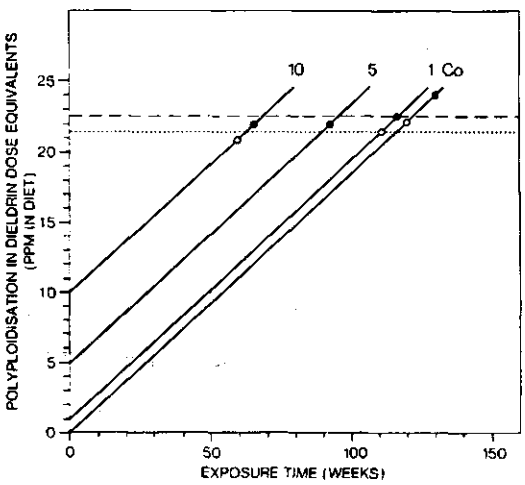

Fig. 5. Polyploidization in mouse liver parenchyma as a function of indicated dietary dieldrin concentrations and time (equation 3). Age-associated polyploidization is expressed in units of dietary dieldrin concentration (p.p.m.), using equation (2). ●, Median liver tumour induction period (= 50% incidence); ○, time to a 10% incidence of liver carcinomas.

Polyploidisation associated with dieldrin exposure as well as with ageing (~ d + ct) is illustrated in figure 5. Ploidy status at the (known) exposure time interval associated with a cumulative frequency of 50 % liver tumours (adenomas or carcinomas) or 10 % liver carcinomas (6) can be estimated for each dietary dieldrin concentration and for non-dieldrin-treated controls. The results are visualised in figure 5, and suggest a virtually constant ploidy status at the time of liver tumour (carcinoma) formation in each group.

DISCUSSION

The enhancement of polyploidisation in CF-1 mouse liver by dieldrin, analyzed in "steady-state" situations was found to be proportional to

dietary dieldrin concentation, and independent of dieldrin exposure time. The absence of an "exposure time" effect on the enhancement of polyploidisation indicates that, in "steady-state", the level of interaction of dieldrin with specific receptors does not change in the course of time. A constant and time-independent level of receptor binding implies, that in "steady state", the velocity of association of dieldrin with specific receptors will be equal to the velocity of dissociation from these receptors (19).

Accordingly, this type of receptor binding is likely to be reversible upon withdrawal and elimination of the compound. This concept is consistent with the available evidence on the reversibility of polyploidisation induced by microsomal enzyme inducers (10,12,20 van Ravenzwaay, unpublished results). Thus, the enhancement of polyploidisation by microsomal enzyme inducers would seem to be determined by the steady state concentration at the site of interaction with specific receptors ("Konzentrationsgift"),

Table II. Age- and dieldrin-related polyploidization as a function of specific receptor binding[a,b]

Polyploidization effector	Reversibility of receptor binding	Receptor binding in relation to compound concentration	Reversibility of the effect	Effect in relation to receptor binding	Effect in relation to compound concentration	Dose–response characteristics
Dieldrin	$T_R - 0$	$C_R \sim C$	$T_r - 0$	$E \sim C_R$	$E \sim c$	Dose-dependent ('Konzentrationsgift')
Age(ing)	$T_R - \infty$	$C_R \sim \int c\,dt$	$T_r - 0$	$E \sim C_R$	$E \sim \int c\,dt$	Dose- and time-dependent ('c.t.-Gift')

[a] T_R = time constant for the reversibility of receptor binding; T_r = time constant for the reversibility of the effect; c = compound concentration at the site of interaction with receptors; C_R = concentration of receptor binding; E = effect, i.e. polyploidization; t = exposure time.
[b] This concept is based on the theories developed by Druckrey and Küpfmüller (19).

Polyploidisation associated with ageing, as assessed in control mice, was found to be linearly related to time. Equation (2) is consistent with the concept that age-associated polyploidisation is a result of irreversible, and, therefore, cumulative interactions of toxic agents with specific receptors (table 2). Age-associated polyploidisation can be regarded

as a function of the product of a constant concentration of toxicants and time ("c.t.-Gift"), as shown in figure 4.

These views are consistent with the progressive nature of age-associated polyploidisation. It is interesting to note, in this context, that the ploidy changes induced in rodent liver by carcinogens, such as dimethylnitrosamine, aflatoxin B_1, and 3'-methyl-4-dimethylaminoazobenzene have been reported to persist after discontinuation of treatment (21,22,23). This evidence may suggest similarities in the mechanisms of age-associated and carcinogen-induced ploidy changes.

Polyploidisation (expressed in ppm dieldrin in the diet) at the median time to liver tumour development (= 50 % incidence) or at the time of a 10 % carcinoma incidence was estimated for each dietary dieldrin concentration and for non-dieldrin-treated controls, and found to be virtually constant across all groups (figure 5). At the time of a 50 % liver tumour incidence there would be a level of polyploidisation of 22.5 ± 0.8 ppm dieldrin in the diet; at the 10 % of a liver carcinoma incidence the level of polyploidisation would be 21.4 ± 0.5 ppm dieldrin in the diet.

The interference that at the time of tumour formation all groups would display the same degree of polyploidisation suggests a strong relationship between tumour formation and polyploidisation. The molecular mechanism is clearly a matter of conjecture, but some aspects can be discussed. The enhancement of polyploidisation by microsomal enzyme inducers, such as dieldrin, may be triggered to meet an increased requirement for particular organ-specific activities (gene dosage) i.e. drug metabolism and SER proliferation.

If the rapid duplication of genetic material during the adaptation of the liver to increased functional demands would be followed by (some) nuclear devisions (and chromosomal redistributions) in the steady-state situation, heterozygous mutations could turn homozygous as has been pointed out by

Kinsella and Radman (24). In this way recessive oncogenic information could become phenotypically manifest (20).

The duplication of genetic information in polyploidisation could also be triggered to save the expression of organ-specific functions from irreversible damage to functional units in the genome, a concept which has been advanced previously by Medvedev (25) and Gahan (26). This concept could be reflected by the observed age-associated increase in polyploidisation.

The effect of dieldrin treatment on liver-tumour formation in CF-1 mice can be viewed as a "time-shift", in "spontaneous" tumour formation (6).

A similar "time-shift" is apparent in the enhancement of polyploidisation by dieldrin (figure 1 and figure 4). Judged by ploidy status, dieldrin appears to create a dose-dependent "time-gap" between chronological and biological age of mouse liver in the initial phases of treatment. The data are consistent with the view that tumour formation is imminent at a constant biological age of mouse liver and that tumour promoters may operate by advancing the biological age of their target organ in the initial phases of treatment. Thus, the close similarities in the kinetics of polyploidisation and tumour formation in livers of CF-1 mice exposed to dieldrin suggest that the analysis of ploidy status may serve as an aid to perspective in assessing risks posed by exposure to liver tumour promoters.

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CHAPTER 2.4

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DIELDRIN INDUCED CHANGES IN ISOENZYME COMPOSITION IN THE LIVERS OF CF-1 MICE

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SUMMARY

The isoenzyme composition of lactic dehydrogenase (LDH), pyruvate kinase (PK) and alanine-amino transferase was determined in the livers of CF-1 mice, exposed to 0, 5 or 10 ppm dieldrin in the diet, over a period of 14 months. This study was carried out to evaluate a previously advanced concept in which the liver tumour promotor dieldrin was though to advance the biological age of CF-1 mouse liver. It was found that oral dieldrin exposure induced a dose-dependent shift towards the fetal types of lactic dehydrogenase and pyruvate kinase, within 1.5 months after initiation of treatment. After the initial shift, no additional dieldrin-dependent changes were found in CF-1 mouse liver throughout the experimental observation period of 14 months. Thus, the initial shifts in isoenzyme composition of LDH and PK would appear to reflect the

adaptation of the liver to increased functional demands imposed by the dieldrin treatment. Within liver nodules, observed after 14 months of 10 ppm dieldrin treatment, for both PK and LDH a further shift towards fetal isoenzymes was observed. No evidence for a gradual shift of isoenzyme activity between liver nodules and normal dieldrin treated liver was found.
The expression of the cytoplasmic A-alanine-amino transferase isoenzyme was found to decrease with age, in untreated control mice. Dieldrin treatment enhanced this process in a dose dependent way. These data suggest that dieldrin treatment can accelerate age-dependent changes in gene-expression. Since the CF-1 mouse strain is characterized by age-related liver tumour development, dieldrin may operate as a liver tumour promotor in these mice by advancing the biological age of its target organ. The observed shift towards fetal isoenzymes may facilitate the expression of the intrinsic neoplastic potential of CF-1 mouse liver.

INTRODUCTION

The CF-1 mouse strain is characterized by the development of "spontaneous" liver tumours when they reach an advanced age. Continuous treatment with microsomal enzyme inducers, such as drugs, food additives and pesticides, enhances liver tumour development in these mice (Thorpe and Walker, 1973; Tennekes et al.,1981). Dieldrin was found to enhance liver tumour formation in CF-1 mice in a dose-dependent way (Tennekes et

al., 1985).

We recently found that nuclear polyploidization of hepatocytes in untreated control CF-1 mice increases proportionally with time. The contribution of dieldrin to nuclear polyploidization consists in a dose dependent enhancement of polyploidization shortly after initiation of treatment. A comparative analysis of polyploidization and liver tumour formation in dieldrin treated CF-1 mice revealed that liver tumour formation would be associated with a constant level of polyploidization (van Ravenzwaay et al., 1985), suggesting a relationship between polyploidization and tumour formation. Since polyploidization is an age dependent process (Shima and Sugahara, 1976) its enhancement by dieldrin might reflect advanced biological ageing. Accordingly it could be hypothesized that tumour promotors, such as dieldrin, could advance the biological age of their target organ and thus accelerate liver tumour formation. The present study was carried out to evaluate this hypothesis.

In many cases total enzyme activity can be attributed to the combined action of its distinct isoenzymes . With regard to this study, it is important to note that different isoenzymes are expressed in the various phases of life (fetal, neonatal, adult and old). Changes in isoenzyme composition could therefore serve as a parameter to elucidate a possible relationship between dieldrin treatment and advanced biological ageing. Over a period of 14 months the isoenzyme composition of three different isoenzyme systems was determined to study this question.

Lactic dehydrogenase (LDH) (EC. 1.1.1.27) has five isoenzymes, H_4 -, H_3M-, H_2M_2-, HM_3-, and M_4 -LDH. These are composed of the two subunits, H and M, which are coded by two separate genes (Lalley et al., 1978; Market, 1968). In fetal liver the M_4 isoenzyme is the predominant form (Farron et al. 1972). In the course of ageing the ratio of M- to H-subunits has been reported to decrease (Singh and Kanungo, 1968). In many neoplastic tissues a shift towards the M isoenzyme has been reported (Fischer et al.,1983).

Different forms of pyruvate kinase (PK) (E.C. 2.7.1.40) named L-, M- and K-PK have been found in mammalian tissues (Ibsen and Trippet, 1972). In normal adult liver L-PK is the prevalent form, though K-PK is also present (Hall and Cottam, 1978). In fetal liver and in hepatocarcinomas K-PK has been shown to be the most active form (Farron et al., 1972; Yanagi et al., 1984). K- and L-PK are produced by two different genes (Moore and Bulfield, 1981; Peters et al., 1981).

Cytoplasmic alanine amino transferase (AAT)(EC. 2.6.1.2) has two isoenzymes (A and B) which are under separate genetic control (Chen and Giblett, 1971). In adulthood both types are present, while in young rats only A-AAT has been found, in old ones only B-AAT (Kanungo et al., 1978).

MATERIALS AND METHODS

Animals

CF-1 mice were kindly provided by Shell Research Ltd, Sittingbourne, Kent, UK. The colony was maintained under SPF

conditions at Ivanovas GmbH, Kieslegg, FRG. Weanling female CF-1 mice were supplied to the German Cancer Research Centre upon request. The animals were allocated to groups and acclimatised for 1 week. Dieldrin treatment commenced at 4-5 weeks of age. The animals were exposed to 0,1,5 and 10 ppm dieldrin in a C-1000 diet (control and experimental diets were prepared by Altromin GmbH, Lage, FRG). Diet and water were given ad libitum.

Preparation of soluble fractions from nodular and non-nodular liver tissues

All chemicals used were obtained from Sigma, with the exception of ADP, NADH and phosphoenol pyruvate which were obtained from Boehringer, Mannheim. Animals were weighed and then killed by cervical dislocation between 9 and 10 a.m. The livers were quickly excised,the gall bladder was removed and the liver tissue was chilled in ice-cold 0.25 M sucrose/ TKM (0.05 M Tris-HCl, 0.025 M KCl and 0.005 M $MgCl_2$, pH 7.4) for a few minutes. One part of the liver was blotted, weighed and, if present, liver nodules were dissected free from the surrounding normal liver tissue. Hereafter liver nodules and normal liver tissue were separately prepared using the same methods for both tissues. The other part of the (normal) liver was used for histochemical analysis. The liver tissues were homogenized with 4 volumes of 0.25 M sucrose/TKM in a Potter-Elvehjem homogeniser (10 passes, 1400 rpm). The homogenate was centrifuged at 104,000xg at 4 C^{o} for 1 h. The resulting supernatant was used for the assays.

Protein content of the supernatant was determined according to Lowrey et al. (1951), using bovine albumine serum (BSA) as a standard.

Assay for pyruvate kinase isoenzymes

The proportion of L- and K-PK was determined by measuring the pyruvate kinase activity in the presence and absence of 5 mM tryptophane, as described by Ibsen et al. (1975). Tryptophane completely inhibits K-PK activity, while L-PK retains 85% of its activity (as measured by purified L-PK). Thus the pyruvate kinase activity in the presence of tryptophane x 1.18 was used to calculate the L-PK activity.

Electrophoresis

Lactic dehydrogenase and alanine-amino transferase isoenzyme composition were determined by means of cellulose acetate (Sartorius) electrophoresis, using a Biotec-Fischer electrophoresis tank. Lactic dehydrogenase electrophoresis and staining were carried out according to Meera Khan (1971), with some minor modifications. Cellulose acetate sheets were incubated in the buffer solution, to which 0.5 mg BSA/ml buffer was added, for 10 min. Circa 200 µg of soluble protein fraction were loaded on the sheets. Electrophoresis was performed for 1.25 h at 60 V. Stained cellulose acetate sheets were subsequently analysed with a thin-layer chromatogram scanner (KM3-Zeiss Instruments) at 510 nm. This instrument was coupled to an integrator (Minigrator, Spectra-Physics) to quantitate the isoenzyme composition. The proportion of M to

H subunits was calculated using the detected isoenzyme activity, and multiplying this activity with the proportion of M and H subunits in the 5 isoenzymes (e.g. M_4 = 100% M-subunit, M_3H= 75% M- + 25% H-subunit etc.).

The expression of cytoplasmic alanine-amino transferase isoenzymes was studied using cellulose acetate electrophoresis. Before electrophoresis, the sheets were incubated in the buffer solution, to which 0.5 mg/ml BSA was added, for 10 min. The buffer solution contained 0.055 M Tris-borate (pH 8.9). Electrophoresis was performed for 1.5 h at 90 V. The staining medium contained 0.1 M KPO_4 (pH 7.3), 10 mM α-ketoglutarate, 10 mM alanine, 4 mM NADH and 15 units lactic dehydrogenase. Staining was carried out in the dark for 16 min at 37 C°. Stained sheets were photographed under the light of a short-wave length light source.

Histochemical analysis

Serial sections of 10 μm were prepared at -15 C°. on a cryostat microtome and used for the enzyme histochemical procedure. Glucose-6-phosphatase activity was demonstrated according to the method of Wachstein and Meisel (1956). Three sections of each liver were projected (magnification 45-fold) and digitized using a manual optic picture analyser (Kontron, Digicon, Munich, FRG.) and the amount of G-6-Pase deficient preneoplastic foci was subsequently quantitated.

RESULTS

Cytoplasmic alanine amino transferase isoenzymes

Adult, 3-month-old untreated control CF-1 mice were found to have two distinct cytoplasmic alanine-amino transferase isoenzymes. At the age of 14 months the slower migrating A-isoenzyme could no longer be detected (Fig. 1).

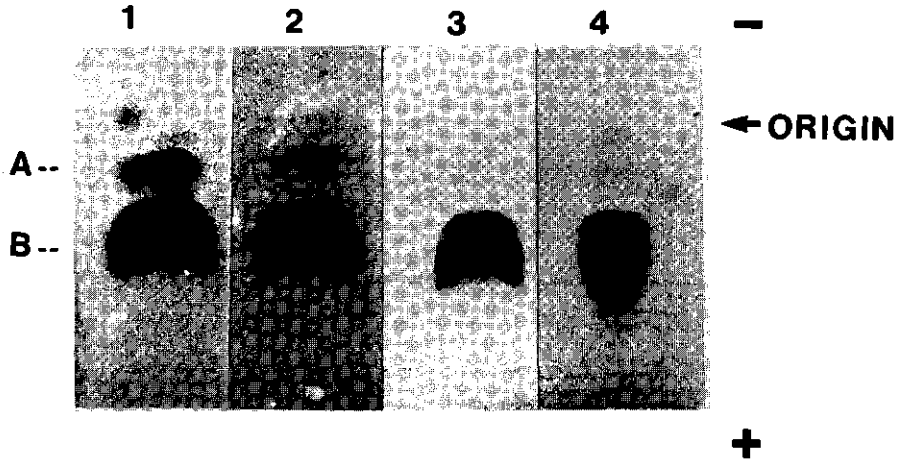

Figure 1 - Electrophoresis of cytoplasmic alanine-amino transferase isoenzymes (A and B) on cellulose acetate from livers of dieldrin treated CF-1 mice.
Lane 1, 3-month-old untreated controls
Lane 2, 3 months treatment, 5 ppm dieldrin
Lane 3, 3 months treatment, 10 ppm dieldrin
Lane 4, 14-month-old untreated controls

These findings are in agreement with the studies of Kanungo et al. (1978). Three months of treatment with 5 ppm dieldrin considerably decreased the activity of the A-isoenzyme. Treat-

ment with 10 ppm dieldrin for the same period of time resulted in a complete disappearance of the A-isoenzyme (Fig.1). Thus, dieldrin treatment seemed to accelerate the age-dependent loss of the A-alanine-amino transferase isoenzyme.

Pyruvate kinase isoenzyme activities

The activity of the L-PK was lowered by dieldrin treatment throughout the whole observation period, proportional to the dietary dieldrin concentration (Table I) . An even more pronounced decrease was seen in liver nodules when compared to the non-nodular surrounding tissue of the dieldrin-treated liver (Table II).

TABLE I - PYRUVATE KINASE ISOENZYME ACTIVITY IN THE LIVERS OF DIELDRIN TREATED CF-1 MICE

		PK activity (nM/mg soluble protein/min)			
Treatment time (months)	PK-isoenzyme	Dieldrin dose (ppm) 0	1	5	10
1.5	L	64.7 ± 8.9	65.4 ± 15.3	57.3 ± 2.4	44.6 ± 7.1^{c}
	K	17.2 ± 4.8	17.2 ± 10.0	19.9 ± 3.8	17.5 ± 6.1
3	L	124.6 ± 19.9	139.9 ± 19.8	118.4 ± 21.5	106.5 ± 24.4^{b}
	K	32.8 ± 8.1	39.0 ± 6.8	43.9 ± 14.1^{b}	50.7 ± 6.0^{c}
6	L	190.0 ± 25.3	199.4 ± 29.5	165.0 ± 26.1^{b}	144.8 ± 17.8^{c}
	K	43.5 ± 8.6	44.9 ± 11.9	62.1 ± 13.2^{c}	72.9 ± 8.6^{c}
9	L	195.8 ± 8.8	-	183.5 ± 10.8^{a}	161.1 ± 12.6^{c}
	K	55.7 ± 12,7	-	68.2 ± 11.4^{a}	76.7 ± 3.9^{b}

n=5 in all cases, exept for 0 ppm at 3 and 6 months where n=10

a: $p<0.05$, b: $p<0.01$, c: $p<0.001$ (significant difference between treatments and control).

The K-PK activity did not change for up to 1.5 months of treatment. After 3 months of treatment, however, dieldrin increased the K-PK activity. This effect appeared to be dose dependent (Table I). In liver nodules, the K-PK activity was found to be substantially higher, than in the non-nodular, dieldrin treated livers (Table II).

TABLE II - PYRUVATE KINASE ISOENZYME ACTIVITY IN NORMAL LIVER TISSUE AND LIVER NODULES FROM CF-1 MICE TREATED WITH 10 PPM DIELDRIN FOR 14 MONTHS

PK activity (nM pyruvate/mg soluble protein/min)		
Nodules	L-PK	K-PK
Small	155.8 ± 36.7	86.1 ± 15.7
Large	160.8 ± 12.0	117.9 ± 11.1
None	181.2 ± 18.0	64.6 ± 15.9

n=4 in all cases

small nodules : weight < 300 mg

large nodules : weight > 500 mg

The L:K-PK ratio did not significantly change in untreated control CF-1 mice throughout the entire observation period of 14 months. However, the L:K-PK ratio was lowered dose-dependently by dieldrin in the initial phases of treatment.

The duration of treatment had no additional effect upon the dieldrin-induced change in the L:K-PK ratio (Fig.2).
In liver nodules a strong decrease in the L:K-PK ratio was observed. These findings suggest that the initial shift reflects an adaptation of the liver to increased functional demands imposed by dieldrin treatment.

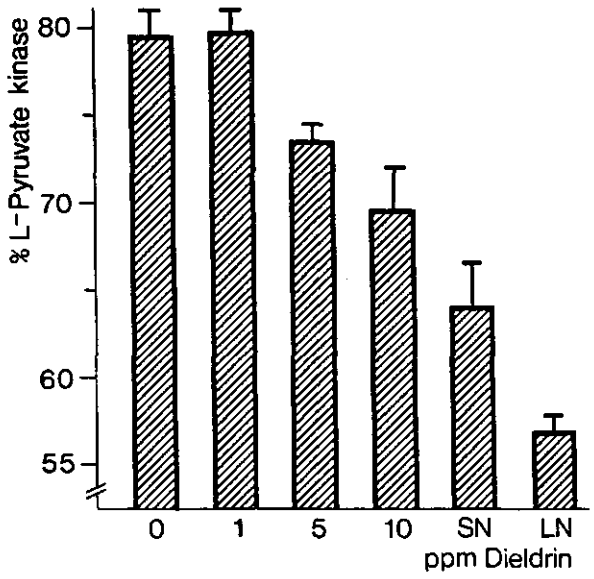

Figure 2 - Relative amount of L-pyruvate kinase, and standard deviation (vertical bars) in the livers of dieldrin-treated CF-1 mice, calculated from Tables I and II.
SN, small nodules (weighing < 300 mg),
LN, large nodules (weighing > 500 mg).

Lactic dehydrogenase subunit composition

In untreated control CF-1 mice an age-dependent decrease of M-LDH subunits was observed. Dieldrin resulted in a dose-

dependent increase of M-LDH subunits in the initial phases of treatment. Thereafter, an age-dependent decrease of M-LDH subunits was observed similar to the one seen in untreated controls (Table III). It would thus appear that the initial shift reflects an adaptation of the liver to the dieldrin treatment in the same way as observed for PK-isoenzymes.

TABLE III - THE PERCENTAGE OF THE M-LACTIC DEHYDROGENASE SUBUNITS IN THE LIVERS OF DIELDRIN TREATED CF-1 MICE

	% M-LDH		
Dieldrin dose (ppm)	Treatment time (months)		
	3 (n)	9 (n)	14 (n)
0	67.0 + 5.7 (7)	65.3 + 2.7 (4)	61.6 + 6.9 (4)
1	71.5 + 6.1 (4)	-	-
5	73.9 + 4.4 (5)[a]	67.4 + 7.8 (4)	-
10	78.3 + 4.0 (5)[c]	71.4 + 4.8 (4)[b]	71.5 + 6.2 (3)[a]
10[d]	-	-	78.5 + 1.5 (3)[c]

n: animal number

a: $p < 0.05$, b: $p < 0.01$, c: $p < 0.001$ (significant difference between treatments and control).

d: liver nodules

In nodular liver tissue the proportion of M-LDH subunits

was considerably increased over normal tissue. Such a shift in neoplastic liver tissue has been reported previously by several authors (Farron et al, 1972; Fischer et al., 1983).

Histochemistry

Changes in the glucose-6-phosphatase activity were used to identify preneoplastic lesion in CF-1 mouse liver, G-6-Pase being the best marker for foci in mouse liver. The amount of G-6-Pase deficient lesions was estimated using serial sections of frozen liver tissue. It was found that, after 14 months of 10 ppm dieldrin treatment in normal liver tissue (i.e. dissected free from nodules) the volume occupied by preneoplastic lesions was: $2.9 \pm 1.6\%$ (n=4). For all other treatment levels, the amount of G-6-Pase deficient foci was negligible, i.e. $< 0.5\%$.

DISCUSSION

A possible mechanism by which dieldrin could operate as a liver tumour promotor might be the advancing of the biological age of mouse liver. This concept has emerged from studies in which a dose dependent enhancement of the age-related process of polyploidization was found in dieldrin treated CF-1 mice (van Ravenzwaay et al., 1987). Moreover, the duplication of genetic material without cellular division has been suggested to be a cause of ageing (Ohno and Yukifumi 1978).

The quantitation of isoenzyme activity offers a quick and

reproducible method to obtain insight into age-dependent changes in gene-expression. In the present hypothesis dieldrin was expected to interfere with age-dependent physiological mechanisms. Alternatively dieldrin could, in principle, exert its influence on isoenzyme activity by a direct allosterical interference with the isoenzymes. Therefore all isoenzyme assays were first carried out in the presence of dieldrin. It was found that dieldrin at a concentration of 10 ppm (the concentration actually found in the liver given an oral exposure of 10 ppm (Tennekes, 1979)) did not have a significant influence on any of the isoenzymes investigated. Moreover, dieldrin is highly lipophilic and can therefore be assumed to be concentrated in the membrane compartment of the cell, whereas the isoenzymes assayed are all found in the cytoplasm. These findings contradict the possibility that dieldrin itself interferes directly with isoenzyme activity. Therefore it seems more likely that the functional commitment imposed on mouse liver by dieldrin may influence existing physiological mechanisms resulting in an altered isoenzyme composition.

The expression of A-alanine amino transferase has been found to decrease with age in the studies of Kanungo et al. (1978) as well as in our studies. Dieldrin treatment resulted in a dose dependent decrease of A-alanine amino transferase expression (Fig.1). The results of the histochemical analysis of the livers showed that the amount of preneoplastic foci was so low that their presence cannot be expected to exert a significant influence on overall isoenzyme composition in

normal CF-1 mouse liver tissue. Thus, these findings are not in variance with the proposed hypothesis that dieldrin treatment may advance the biological age of CF-1 mouse liver.

In contrast, dieldrin treatment results in a dose dependent shift in the expression of lactic dehydrogenase and pyruvate kinase isoenzymes towards the fetal types, in the initial phases of treatment. The duration of the treatment was found to have no additional effect on isoenzyme composition. In the case of LHD the age dependent decrease of M-subunits was the same in dieldrin treated groups and in untreated controls (Table III). The L:K-PK ratio did not change at all with age (Fig 2). The lack of any influence of the length of exposure time suggests that a "steady-state" situation is maintained. Thus, the initial shift induced by dieldrin would appear to be an adaptation of the liver to increased functional demands.

A further shift towards fetal isoenzymes was observed in liver nodules. These findings are not in variance with the reported fetal character of (liver) tumours (Farron et al., 1972).

In conclusion, it would seem that dieldrin treatment can advance age-dependent changes in isoenzyme expression. It is conceivable that advancing the biological age of a mouse strain, prone to "spontaneous" age-related liver tumour formation, may result in an enhancement of liver tumour development in these mice.

The paradox of advanced biological ageing and the induced shift towards fetal isoenzymes both resulting from

dieldrin treatment may be solved by a concept put forward by Jose Uriel (1976) : retrodifferentiation (the shift towards fetal isoenzymes) seems to be an alternative to the cell's risk of undergoing irreversible changes when their homeostatic barriers are surpassed (i.e. the adaptation of the liver to dieldrin in the initial phases of treatment). The long-term effects of dieldrin treatment (advancing age-dependent processes), which occur in "steady-state" situations, would seem to be of a different nature. These long-term effects are more likely to be involved in the enhanced liver tumour formation in dieldrin treated CF-1 mice.

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CHAPTER 2.5

ACCELERATED NUCLEAR POLYPLOIDIZATION AND TUMOUR FORMATION IN THE LIVERS OF DIELDRIN TREATED CF-1 MICE

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ABSTRACT

Nuclear polyploidization in the livers of CF-1 mice, exposed to dieldrin (0, 1, 5 and 10 ppm in the diet), was studied up to the median time to liver tumour development (ranging from 15 to 27 months) in the respective treatment groups. In untreated controls nuclear polyploidization is characterized by a linear increase of octaploid nuclei with age. Approximately 4 months before tumour development a reduction in the tetraploid to diploid ratio is observed. Dieldrin treatment was found to enhance nuclear polyploidization in the initial phases of treatment, as expressed by a dose-dependent increase in octaploid nuclei. In "steady-state" situations all age dependent changes in the level of polyploidization found in controls were also found in dieldrin treated mice. However, these changes occurred at an increasingly earlier age with higher dieldrin treatment levels. The decrease in

the tetraploid:diploid ratio always takes place a few months before tumour development. This change in the ploidy level may thus be related to the subsequent liver tumour formation. The liver tumours themselves appear to originate from a diploid stem line, and were found to increase their degree of polyploidization during growth, eventually developing aneuploid nuclei. A comparison of nuclear polyploidization and liver tumour formation in CF-1 mouse liver for the given dietary dieldrin concentrations showed that liver tumour formation was associated with a constant level of polyploidization. Since polyploidization is an age-depending process, these findings suggest that liver tumour formation is imminent at a constant biological age and that dieldrin may advance the biological age of CF-1 mouse liver.

INTRODUCTION

The CF-1 mouse strain is characterized by the development of "spontaneous" liver tumours when they reach an advanced age. Continuous treatment with microsomal enzyme inducers, such as drugs, food additives and pesticides, results in an induction of liver microsomal enzyme systems, liver enlargement and an increase in total liver DNA in the initial phases of treatment (1-3). Thereafter a "steady-state" situation is maintained. The induced changes are reversible upon withdrawal and elimination of the compound and are not accompanied by evidence of liver damage. Thus, these changes are likely to be an adaptation of the liver to increased functional demands. However, exposure to microsomal enzyme inducers, such as

dieldrin has been shown to enhance liver tumour formation in these mice (4-6).

Microsomal enzyme inducers are also known to enhance nuclear polyploidization in rodent liver (7,8). In a recent study (9) it was reported that nuclear polyploidization in livers of CF-1 mice increased proportionally to the dietary dieldrin concentration within a few weeks after the initiation of treatment. In "steady-state" situations only an age-dependent increase in nuclear polyploidization was found, which exhibited an equal velocity in all treatment groups including controls. An estimation of the mean level of nuclear polyploidization (employing the linear regression of the data) at the median time to liver tumour development (= 50% incidence) revealed that this level should be the same across all groups.

The objectives of the present study were to ascertain whether or not the degree of polyploidization at the median tumour induction period would be equal across all doses. In our previous report polyploidization was quantitated by the proportion of octaploid (8c) and 16c nuclei only. This study reports the age and dose dependent changes of the other ploidy classes, diploid (2c) and tetraploid (4c), as well as the 8c and 16c nuclei. Furthermore, the level of nuclear polyploidization in the dieldrin induced liver tumour was determined. The greatly reduced glucose-6-phosphatase (E.C. 3.1.3.9) enzyme activity in liver nodules was used to ascertain the presence of preneoplasic foci in sections of normal liver tissue.

MATERIALS AND METHODS

Chemicals. The fluorochrome DAPI was obtained from Serva, Heidelberg, FRG. Glucose-6-phosphatase was obtained from Boehringer, Mannheim, FRG. All other chemicals were purchased from Sigma Chemical Co., Munich, FRG.

Animals. CF-1 mice were kindly provided by Shell Research Ltd, Sittingbourne, Kent, UK. The colony was maintained under SPF conditions at Ivanovas GmbH, Kieslegg, FRG. Weanling female CF-1 mice were supplied to the German Cancer Research Centre upon request. The animals were allocated to groups and acclimatised for 1 week. Dieldrin treatment commenced at 4-5 weeks of age. The animals were exposed to 0, 1, 5 or 10 ppm dieldrin in a C-1000 diet (control and experimental diets were prepared by Altromin GmbH, Lage, FRG). Diet and water were given ad libitum. To determine polyploidization between 5 and 11 animals/group were killed at the indicated exposure time.

Isolation of Liver Nuclei. Animals were weighed and killed by cervical dislocation in "steady-state" situations (i.e. not before 8 weeks after the initiation of treatment). Livers were quickly excised, the gall-bladder was removed and the tissue was weighed. The livers were chilled in ice-cold 0.25 M Sucrose/TKM (0.05 M Tris-HCl, pH 7.4, 0.025 M KCl and 0.005 M $MgCl_2$), for a few minutes. If tumours were present, livers were then dissected free from observed nodules and tissues were weighed. For both tissues one part was used to isolate nuclei, the other part was used for histochemical analysis.

Liver nuclei were isolated according to Blobel and Potter (10). Nuclear pellets were resuspended in 0.35 ml TKM buffer, and fixed by injection into tubes containing 12 ml absolute ethanol at -20 C°.

Flow Cytometry. DNA analysis was performed using 4'-6'-diamidino-2-phenylindole dihydrochloride (DAPI) as the quantitative fluorochrome (11). Flow cytometry was carried out as reported previously (9). In each case 40 000 nuclei were measured. The percentages of diploid and polyploid nuclei were corrected for doublets and higher aggregates of nuclei according to Beck (12).

Histochemical Analysis. Serial sections of 10 µm were prepared at -15 C° on a cryostat microtome and used for the enzyme histochemical procedure. Glucose-6-phosphatase activity was demonstrated according to the method of Wachstein and Meisel (13). Three sections of each liver were projected (magnification x 45) and digitized using a manual optic picture analyser (Kontron, Digicon, Munich, FRG) and the proportion of G-6-Pase deficient preneoplastic foci was subsequently quantitated.

RESULTS

Nuclear Polyploidization in Non-Nodular Liver Tissue. In the liver of CF-1 mice three distinct ploidy classes could be found during the entire observation period: diploid, tetraploid and octaploid nuclei. Nuclei of an even higher ploidy level e.g. 16c were found in aged mice, their proportion remained low (<3.5%), however. Polyploidization in the livers of untreated control CF-1 mice was

found to be determined by two phenomena. Up until the age of 14 months nuclear polyploidization was characterized by a slight decrease of the proportion of diploid (2c) and tetraploid (4c) nuclei. Between 14 and 25.5 months the proportion of 4c nuclei decreased at a higher velocity. Concomitantly, the proportion of 2c nuclei did not decrease any further or even increased somewhat. The percentage of octaploid (8c) nuclei was found to increase linearly with age during the entire experimental observation period (Fig. 1).

Continuous feeding of dieldrin at dietary concentrations of 1, 5 and 10 ppm was found to enhance the proportion of 8c nuclei linearly with the treatment level in the initial phases of treatment, as reported previously (9). In these phases the percentages of 2c and 4c nuclei decreased slightly with increasing dieldrin dose.

The percentage of 8c nuclei was found to increase proportionally with age, the velocity of this process being the same in all treatment groups including controls, until liver tumour formation (i.e. death). In untreated controls the experiment was terminated after 25.5 months (i.e. 4.5 months before the median time to liver tumour development in this group) because the number of mice surviving until 30 months was not expected to be high enough to determine polyploidization. Therefore no data for polyploidization were obtained at the median time to liver tumour development in untreated control CF-1 mice.

In all treatment groups an age-dependent decrease of the

proportion of 2c and 4c nuclei was found, similar to the one observed in controls. Also similarly to controls, the loss of 4c nuclei increased in the later phases of life and continued to increase until liver tumour development. During these months the percentage of 2c nuclei was found to increase (Figs. 2-4). The induction of the change in the 4c:2c ratio appeared to be dose-dependently advanced in time by the dietary dieldrin concentration. In controls the change in the 4c:2c ratio was first observed after 25.5 months, with a treatment of 1 ppm dieldrin after 21.5 months, with 5 ppm after 14 months and with 10 ppm after 9 months (Figs. 1-4).

At the median time to liver tumour formation polyploidization was found to be approximately the same in all treatment groups (Table 1).

Extrapolation of the linear regression of the percentage of 8c nuclei against time for untreated controls (experimental observation until 25.5 months) to the median time of tumour development in this group (30 months) gives an expected 8c proportion of 21.8 % . This value is in agreement with the ones obtained for dieldrin-treated CF-1 mice (Table 1), and emphasizes that liver tumour formation is associated with a constant level of polyploidization.

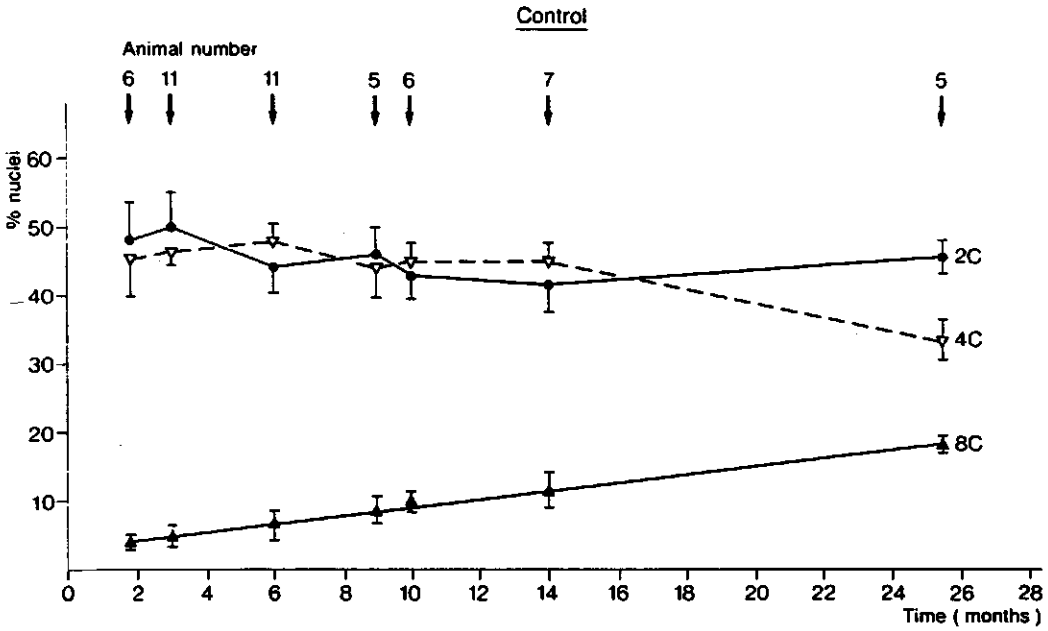

Fig. 1. Mean percentages and standard deviation of diploid (2c), tetraploid (4c) and octaploid (8c) liver nuclei in control CF-1 mice.

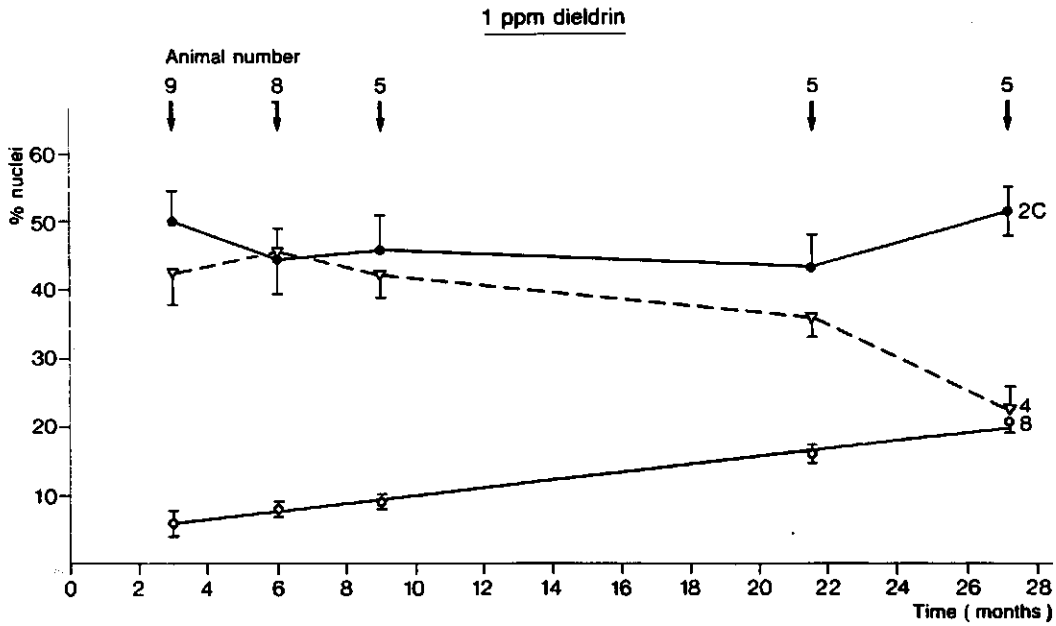

Fig. 2. Mean percentage and standard deviation of diploid (2c), tetraploid (4c) and octaploid (8c) liver nuclei in CF-1 mice treted with 1 ppm in the diet.

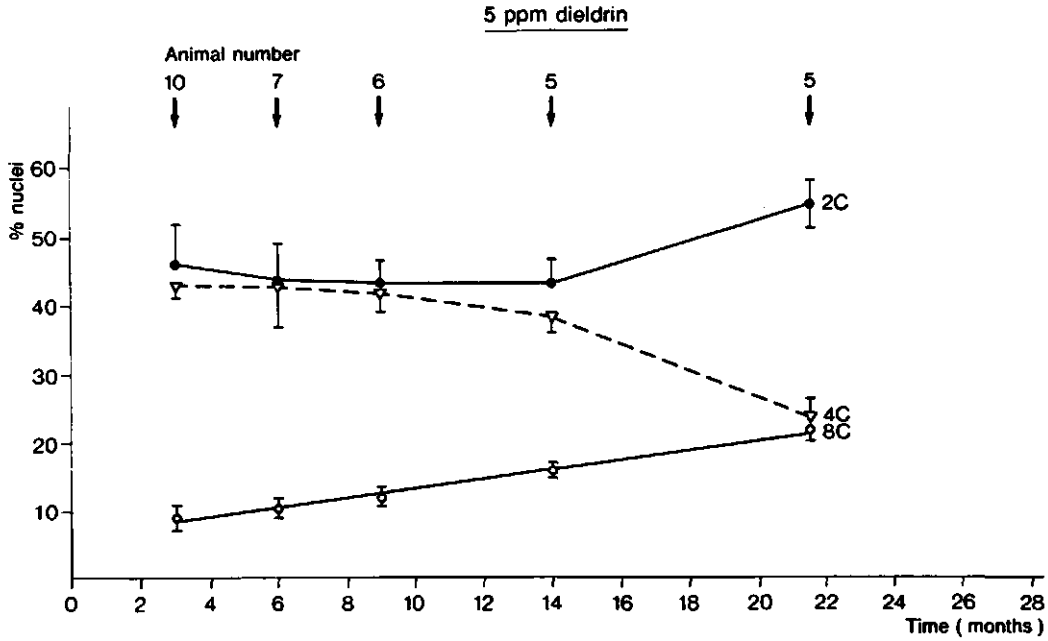

Fig. 3. Mean percentage and standard deviation of 2c, 4c and 8c liver nuclei in CF-1 mice treated with 5 ppm dieldrin in the diet

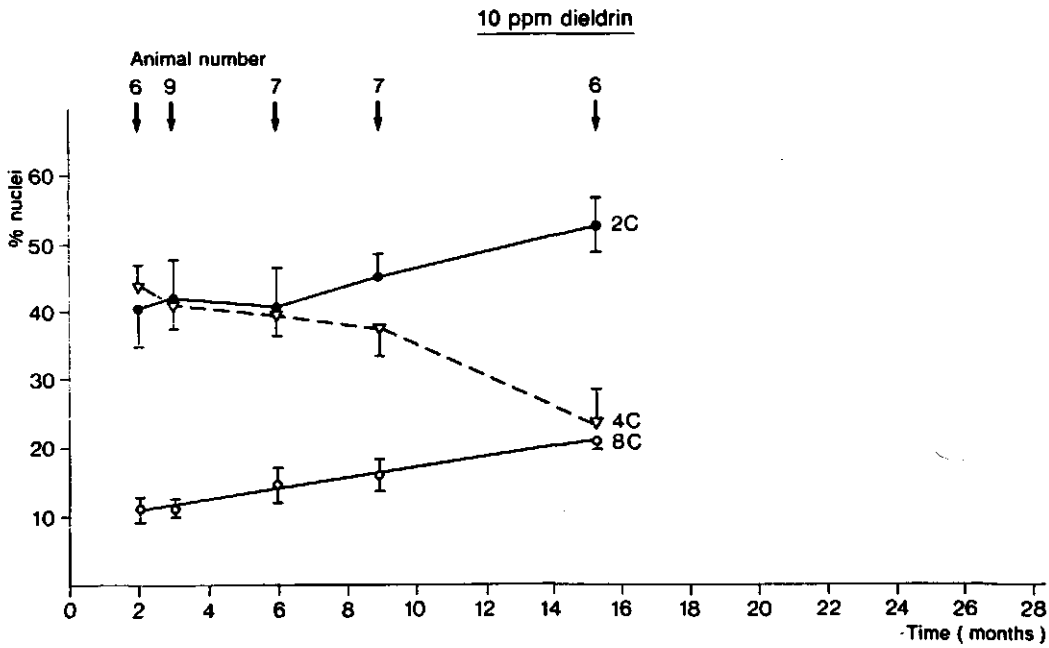

Fig. 4. Mean percentage and standard deviation of 2c, 4c and 8c liver nuclei in CF-1 mice treated with 10 ppm dieldrin in the diet

Table 1. Nuclear polyploidization at the median time to liver tumour formation in CF-1 mice exposed to a dietary dieldrin concentration of 1, 5 and 10 ppm[a]

Dieldrin Dose (ppm)	Median Liver Tumour Induction Period (months)	Percentage 2c	4c	8c
1 (5)	27.25	51.6 ± 3.8	22.4 ± 2.8	21.0 ± 2.2
5 (5)	21.5	54.6 ± 3.5	23.6 ± 3.0	21.2 ± 2.0
10 (6)	15.25	52.5 ± 4.1	23.4 ± 5.5	20.8 ± 1.0

a: number of mice used is indicated in parentheses.

Nuclear Polyploidization in Liver Nodules. Liver nodules, dissected free from the surrounding normal liver tissue were stained for glucose-6-phosphatase activity. G-6-Pase negative nodules (Fig. 5) were divided into three groups according to their weight and were subsequently analysed for polyploidization.

Liver nodules with a weight ‹300 mg contained mainly 2c nuclei. With increasing weight the proportion of 2c nuclei decreased and the proportion of 4c nuclei increased. In nodules weighing ›500 mg even 8c nuclei were found (Table 2). In liver nodules weighing ‹500 mg no aneuploid nuclei could be detected. However, the large nodules (›500 mg) contained approximately 7% aneuploid nuclei (Table 2). Interestingly, all of these nodules contained a aneuploidy class of approximately 2.8c. Some of the nodules were found to have additional aneuploidy classes of 1.6c and 5.0c.

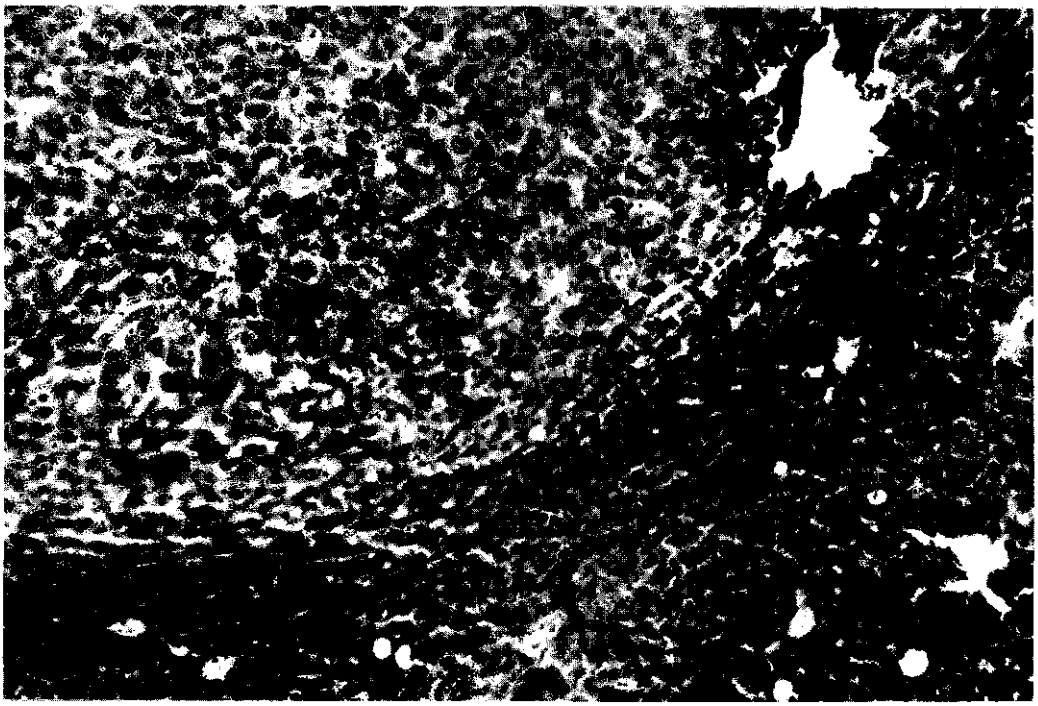

Figure 5. Glucose-6-phosphatase negative liver nodule.

Quantitation of Preneoplastic Foci. Slices of non-nodular liver tissue were stained for glucose-6-phosphate activity to ascertain whether or not microscopic preneoplastic foci were present. If present, the amount of G-6-Pase negative foci was quantitated. It was found that foci in non-nodular liver tissue could be detected only at the end of the dieldrin exposure time. The amount of G-6-Pase negative foci was highest in the 10 ppm treatment group after 14 months of exposure: 2.9 $\pm$ 1.6 % (n=4). After 21 months of 5 ppm dieldrin exposure the amount of foci was 1.32 $\pm$ 0.31 % (n=4); in the 1 ppm group after 27.25 months, 2.00 $\pm$ 0.82 % (n=4); and in untreated controls after 25.5 months, 0.65 $\pm$ 0.35 % (n=4). In all other cases the amount of G-6-Pase deficient foci was negligible.

Table 2. Nuclear polyploidization (%) in glucose-6-phosphatase negative liver nodules taken from CF-1 mice treated with 5 and 10 ppm dieldrin in the diet[a]

Ploidy Class	Tumour Weight		
	< 300 mg (3)	300-500 mg (4)	>500 mg (4)
2c	82.3 ± 3.1 (%)	65.1 ± 9.1	40.3 ± 16.5
4c	15.5 ± 4.8	32.3 ± 6.8	40.5 ± 16.4
8c	0.0	0.0	10.8 ± 7.2
aneuploidy	0.0	0.0	7.2 ± 5.9[b]

a: number of nodules used to determine nuclear polyploidzation is indicated in parentheses

b: aneuploidy classes found were : 1.6c, 2.8c, and 5.0c

DISCUSSION

The quantitation of glucose-6-phosphatase negative preneoplastic foci in non-nodular liver tissue showed that the volume occupied by these foci was very low (<2.9 % in all cases). Their presence thus cannot be expected to have a significant impact on the results of the determination of nuclear polyploidization in non-nodular liver tissue.

The percentage of 8c nuclei in the liver of CF-1 mice was found to be dose-dependently enhanced during the initial phases of dieldrin treatment. In "steady-state" situations the percentage of 8c nuclei increases linearly with age. At the median time to liver tumour

development the mean value of 8c nuclei, for all treatment groups including controls, was 21.3 ± 0.53%. These findings confirm our previous extrapolations (9) which were based on observations until 14 months of treatment. In our earlier report (9) it was proposed that liver tumour formation was imminent at a constant biological age of mouse liver. Dieldrin may thus operate as a tumour promotor by advancing the biological age of the liver in a mouse strain prone to age-related "spontaneous" liver tumour formation. The results of this study further emphasize this concept. As shown in Figs. 1-4, the process of polyploidization (i.e. the kinetics of 2c, 4c and 8c nuclei) observed in untreated controls can also be found in dieldrin treated CF-1 mice but at an increasingly earlier age with higher dietary dieldrin concentrations. The time-gaps created by dieldrin between the biological and chronological age of CF-1 mouse liver for both liver tumour formation (6) and nuclear polyploidization turned out to be virtually the same. The observed quantitative relationship between the degree of nuclear polyploidization and liver tumour formation leads to the question as to whether or not a causal relationship between nuclear polyploidization and tumour formation exists. An interesting feature in the observed kinetics of nuclear polyploidization is the decrease in the 4c:2c ratio. The onset of this decrease occurs approximately 4 months before the median time to liver tumour development in all treatment groups including controls. A decrease in the 4c:2c ratio during carcinogenesis is not an entirely new observation. Neil et al. (14) have found that the administration of aflatoxin B1 resulted in a decrease of 4c nuclei. Styles et al. (15) have also reported a decrease in the 4c:2c ratio when rats

were exposed to the liver carcinogen 3'-methyl-4-dimethylaminoazo-benzene. Moreover, it has been reported that 4c nuclei bind more than twice the amount of carcinogen than 2c nuclei (16). It would appear that 4c nuclei are more sensitive than other ploidy classes.

The fate of the disappearing 4c nuclei is not yet known, however, two mechanisms can be proposed and their implications for hepatocarcinogenesis discussed.

1) Tetraploid cells could, assuming that they are more sensitive to toxicity than other ploidy classes, die when (cumulative) toxic stress goes beyond their homeostatic barriers. Since measurements were performed during "steady-state" situations, i.e. with no gross increase or decrease of liver weight, the necrotic 4c nuclei must be replaced by 2c nuclei (the kinetics of 8c nuclei are not affected by the change in the 4c:2c ratio). The reduction of 4c nuclei ranges between 15%-20% of the total amount of liver nuclei. Since tetraploid nuclei and cells are twice the size of diploid ones (17), two diploid cells have to divide to replace one tetraploid cell. Therefore, the observed decrease in the 4c:2c ratio should result in a strong proliferative signal in the diploid population. By this mode of action the intrinsic neoplastic potential of CF-1 mouse liver may be activated resulting in liver tumour formation.

2) A decrease in the 4c:2c ratio could also occur if the percentage of tetraploid cells were reduced by amitotic nuclear division as observed for polyploid rat liver nuclei (18) and for rabbit trophoblasts (19). In such a case several mechanisms could explain the subsequent tumorigenesis. a: Spontaneous mutations may be duplicated by polyploidization resulting in a heterozygous

situation -MMmm-. Nuclear division of such a tetraploid cell, would, by means of random segregation of chromosomes, result in the occurrence of some diploid -mm- cells, homozygous for the carcinogenic mutation, a concept which has been advanced previously (20,21). b: Since males, females and their offspring are all characterised equally by the development of "spontaneous" liver tumours (4,22) it could be suggested that the neoplastic factor is present in a homozygous form in all CF-1 mice. Since dieldrin has been shown to be devoid of any mutagenic potential (23,24) it would thus appear that the intrinsic neoplastic factor must be activated in order to induce liver tumours. An amitotic division of tetraploid cells could give rise to chromosomal rearrangements favourable for subsequent tumour formation. At least two mechanisms could account for the activation of the neoplastic factor. Translocation of this factor could enhance its expression in the same way as has been reported for the c-myc oncogene in human lymphomas (25,26). Events leading to an amplification of the neoplastic factor (e.g. unequal sister chromatid exchange between homologous chromosomes), could disturb the balance between expressors and suppressors, resulting in tumour development (27). c: By the same mode of action a factor repressing the expression of the neoplastic factor may be inactivated or diluted, with subsequent tumorigenesis. Impaired expression of a tumour suppressing gene has been demonstrated to be involved in tumour development in the case of human retinoblastomas (28) and Wilm's tumours (29).

The results of the determination of nuclear polyploidization in liver nodules show an increasing occurrence of polyploid nuclei

with increasing weight (i.e. age) of nodules (Table 2). Medvedev and Medvedeva (30) have also reported that nuclei with a high ploidy level were found only in the larger "spontaneous" hepatocarcinomas of CBA mice. In this report it has been shown that in the largest liver nodules approximately 7% of all nuclei were aneuploid. Aneuploidy is generally regarded as a situation indicating malignancy. It has indeed been found that the "spontaneous" liver tumours of CF-1 mice do become malignant and metastasize, as shown in Fig. 6. The shift from 2c to 4c and 8c nuclei in liver nodules with increasing weight (Table 2) suggests that the origin of the liver nodules may be found in the diploid population. This would be in agreement with the proposed mechanisms for the activation of the intrinsic neoplastic potential of CF-1 mice, which all implicate the diploid population as the source of CF-1 mouse liver tumours.

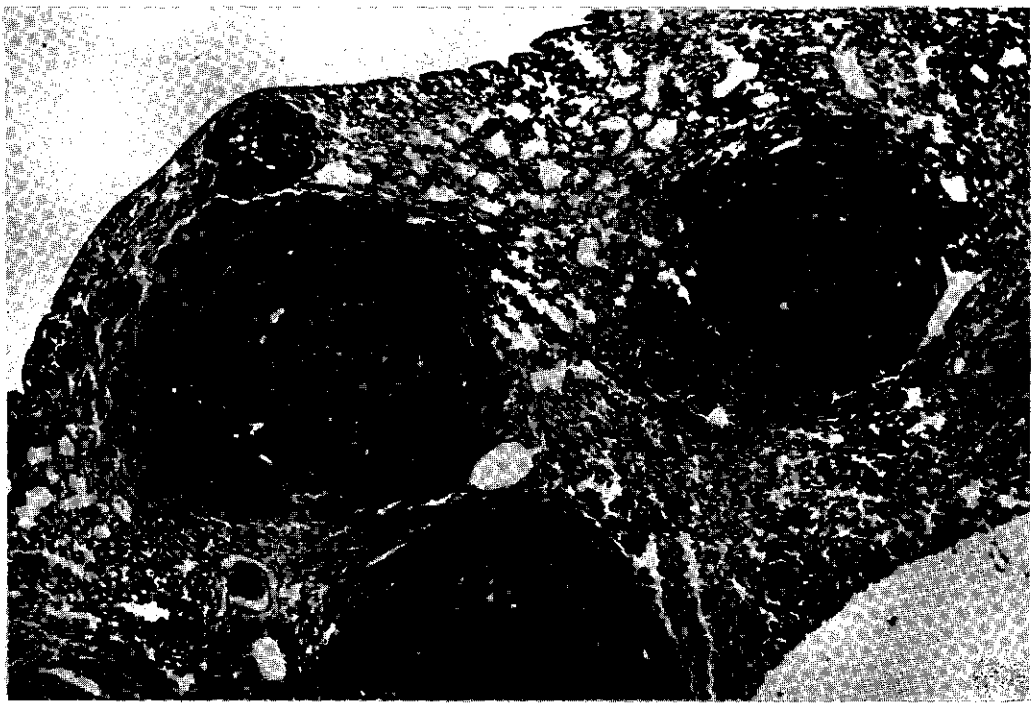

Figure 6. Metastasis of a liver tumour in CF-1 mouse lung

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CHAPTER 2.6

POLYPLOIDIZATION AND ITS POSSIBLE ROLE IN HEPATOCARCINOGENESIS: STUDY WITH ^{3}H-THYMIDINE-LABELLED NUCLEI IN DIELDRIN-INDUCED HEPATIC NUCLEAR POLYPLOIDIZATION

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(submitted to Carcinogenesis)

Exposure to a variety of xenobiotic compounds has been shown to enhance liver tumour formation in mouse strains susceptible to "spontaneous" liver tumour formation (1-3). Moreover, these compounds have also been shown to promote the formation of rat liver tumours from lesions previously initiated by hepatocarcinogens (4-5). It has been suggested that these xenobiotics, which are inducers of microsomal enzyme activity, increase the functional pressure on the liver and may thus facilitate the expression of its intrinsic neoplastic potential (1,6). Nuclear polyploidization has also been shown to be related to the level of functional commitment (7-9). In our previous report we have demonstrated that liver tumour formation in CF-1 mice, exposed to various concentrations of dieldrin, is associated with a constant level of polyploidization (10), suggesting a relationship between polyploidization and liver tumour formation. Concerning the nature of this relationship we proposed that if the duplication of genetic material were to be followed by some (amitotic) nuclear divisions (and chromosomal redistributions), then heterozygous mutations could turn homozygous, a concept originally put forward by Kinsella and Radsman (13). In our earlier report (10) nuclear polyploidization in dieldrin-treated CF-1 mouse liver was determined for a period of 14 months. Recent findings demonstrated a decrease in the

tetraploid-diploid ratio of hepatocyte nuclei approximately 4 months before tumour development in all treatment groups (van Ravenzwaay, unpublished results). These findings may be associated with a carcinogenic mechanism (11), and they emphasized the possible role of amitotic nuclear divisions (12).

The objectives of the present study were to ascertain whether amitotic nuclear divisions in hepatocytes could be detected.

Eight-week-old, male, CF-1 mice (kindly provided by Shell Research Ltd, Sittingbourne, Kent, U.K.) were exposed to 0 or 10 ppm dieldrin in the C-1000 diet (prepared by Altromin GmbH, Lage, FRG). Five hours after the initiation of the experiment (i.e. 2 p.m.), all mice of both treatment groups received a subcutaneous injection of ^{3}H-thymidine (Amersham) with an activity of 5 µCi. Three days later this procedure was repeated. Four weeks after initiation of the experiment, five animals of the control and 10 ppm dieldrin groups were killed by cervical dislocation, and the remaining mice of the 10 ppm treatment group were put on a control diet thereafter. Three weeks later these animals were also killed. Liver nuclei were isolated as described by Blobel and Potter (14). Nuclear pellets were resuspended in 0.35 ml TKM [0.05 M Tris-HCl, pH 7.4 (20°C), 0.025 M KCl and 0.005 M $MgCl_2$] buffer and fixed.

Determination of nuclear polyploidization and sorting were carried out by double staining with DAPI (4'-6-diamidino-2-phenylindole dihydrochloride) and SR_{101} (sulphorhodamine) using the HEIFAS cell sorter. This system has been described elsewhere (15). For nuclear polyploidization determination 40 000 nuclei were measured; the determination of ^{3}H-thymidine activity was done with no less than 350 000 nuclei/animal in each ploidy class.

The ^{3}H-thymidine activity (expressed in CPM/100 000 nuclei for each ploidy class) in untreated controls four weeks after the ^{3}H-thymidine application did not differ among the three ploidy classes, the activity

being somewhat lower in 8c groups. Four weeks' exposure to 10 ppm dieldrin resulted in an increase in relative liver weight (+ 38%). This increase was found to be associated with polyploidization, resulting in an augmentation of the proportion of 8c nuclei, and a smaller one of the 4c nuclei. The ^{3}H-thymidine activity in the 10 ppm treatment group had increased slightly in the 2c group and remained virtually constant in the 4c group. In contrast, there was a profound increase of ^{3}H-thymidine activity in the 8c group (+ 42%). These results suggest that DNA synthesis, associated with dieldrin-induced liver enlargement, can be attributed to the increased formation of 8c nuclei in hepatocytes.

Table 1. ^{3}H-thymidine activity and percentage of nuclear polyploidization for each ploidy class in dieldrin-treated CF-1 mouse liver

	0 ppm (4 weeks)	10 ppm (4 weeks)	"cessation" (3 weeks)
2c	35.7 ± 2.5%	32.3 ± 3.2%	40.8 ± 2.3%
^{3}H	65.9 ± 10.5[a]	70.5 ± 10.2	82.9 ± 8.9
4c	41.8 ± 3.1%	43.2 ± 4.1%	35.8 ± 1.6%
^{3}H	70.1 ± 13.6	69.1 ± 18.7	56.6 ± 11.5
8c	13.7 ± 2.9%	19.2 ± 1.7%	15.9 ± 2.3%
^{3}H	58.8 ± 10.9	83.4 ± 13.1	58.8 ± 7.4
RLW	5.04 ± 0.26	6.93 ± 0.35	5.28 ± 0.29

a: activity is expressed as CPM per 100 000 nuclei per ploidy class
n = 5; RLW = relative liver weight

Three weeks after cessation of treatment, relative liver weight had decreased to near control value. Dieldrin-induced polyploidization was also found to be reversible after discontinuation of treatment. The proportion of 8c nuclei had decreased but was still somewhat higher than

in untreated controls. However, the proportion of 8c nuclei in untreated controls was determined three weeks before the one in the "cessation" group. Since the percentage of 8c nuclei in CF-1 mice increases proportionally with time (10), the actual difference would, in reality, be smaller. The proportion of 4c nuclei was also found to be decreased; in this case the mean value of the "cessation" group was lower than in untreated controls. In contrast with the 4c and 8c nuclei, the proportion of 2c nuclei had increased (+ 26%) to a level higher than in untreated controls.

The ^{3}H-thymidine activity in the 8c group had returned to control value three weeks after cessation of treatment. In the 4c group, activity also declined. In comparison, the activity in the 2c groups was found to be increased after a three-week period of control diet feeding.

The results of the present study demonstrate the reversibility of dieldrin-induced liver enlargement (as expressed in terms of relative liver weight) and nuclear polyploidization. The expected reversibility of both dieldrin-induced liver enlargement (1,2) and nuclear polyploidization (van Ravenzwaay, unpublished observations) were important features in the rationale for experimentation. The induction of liver enlargement and nuclear polyploidization by dieldrin were expected to result in increased ^{3}H-thymidine activity in the 8c group. This can be demonstrated even more clearly by calculating the average ^{3}H-thymidine activity per 100 000 nuclei, as related to liver weight. In untreated controls average ^{3}H-thymidine activity was 60.9 cpm; after 4 weeks of 10 ppm dieldrin in the diet the calculated activity was 101.8 cpm. Since ^{3}H-thymidine was applied in two "pulses" at the beginning of the experiment, no free ^{3}H-thymidine was to be expected after 4 weeks of treatment. Thus, if the reversibility of liver enlargement and nuclear polyploidization were brought about by (controlled) cell death alone, only a decrease in the specific ^{3}H-thymidine activity in these polyploid nuclei

is to be expected. If, however, some amitotic nuclear divisions of higher polyploid nuclei occurred during the regression phase, an increase in specific ^{3}H-thymidine activity in the lower ploidy (2c or 4c) groups should be detectable.

The increased ^{3}H-thymidine activity in the 2c groups (Table I) suggests that the reversibility of nuclear polyploidization can partly be attributed to the occurrence of amitotic nuclear divisions. It is interesting to note that the increased levels of ^{3}H-thymidine activity found in the 8c group after four weeks of dieldrin treatment returned to control values three weeks after cessation of treatment. These findings suggest a "memory-effect" in the reversibility of nuclear polyploidization, i.e. the 8c nuclei formed during the adaptation of the liver to dieldrin exposures are the ones that disappear during regression. The inference can be made that the recessive oncogenic potential of CF-1 mouse liver is activated by a genetic mechanism.

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3 DISCUSSION

The apparent paradox between the reversibility of dieldrin-induced changes in the livers of CF-1 mice (liver enlargement, microsomal enzyme induction and proliferation of the smooth endoplasmic reticulum) and the irreversibility of dieldrin enhanced liver tumour formation may be considered as the main problem on which the studies presented in this thesis were focused. The tumorigenic effects of microsomal enzyme inducers, such as dieldrin, can be explained by assuming that:

a) dieldrin possesses a weak carcinogenic activity itself, or,

b) dieldrin promotes the expression of an intrinsic neoplastic factor in CF-1 mouse liver.

In this thesis it is demonstrated that the dose-response characteristics of dieldrin enhanced liver tumour formation can be expressed as:

$$(do + \delta x) \times t = \text{constant} \qquad (1)$$

where do = the background dose equivalent for the induction of "spontaneous" liver tumours in control CF-1 mice, δx = the actual dieldrin dose (ppm in the diet) and t = the median tumour induction period in CF-1 mice treated at a dieldrin level δx. This relationship is a Druckrey equation, with a reinforcement factor $n=1$. Since all chemical carcinogens have been shown to exhibit dose-response relationships where $n > 1$, this finding suggests that dieldrin cannot be considered as a weak carcinogen but exerts its tumorigenic action by enhancing a pre-existing oncogenic factor (i.e. tumour promotion). Druckrey and Küpfmüller (1949) have elucidated that a reinforcement factor $n > 1$ is associated with irreversibility of receptor binding and irreversibility of the effect of receptor binding. A dose-response relationship where $n = 1$, such as the liver tumour enhancing effect of dieldrin, is associated with either irreversibility of receptor binding or with irreversibility of the effect of

receptor binding. There is evidence to indicate that the numeric value of the reinforcement factor (n) parallels the initiating capacity of the carcinogen (Schwarz et al., 1984). Since dieldrin and its metabolites have been shown to be devoid of any genotoxic potential (Dean and Doak, 1975; Bidwell et al., 1975), the observed value of n (=1) in the dieldrin study suggests that dieldrin binding to its receptors is reversible, whereas the effect of the receptor binding on the neoplastic process is irreversible. This inference is further confirmed by the complete reversibility of dieldrin-induced subcellular changes (Chapter 3.2).

Moreover, it is demonstrated that the liver tumour-enhancing effects of (limited) treatment with dieldrin followed by a treatment free period, and a treatment free period followed by dieldrin application are consistent with equation (1). These findings indicate that:

1) Liver tumour formation is associated with a constant total tumorigenic dose.
2) There is no evidence for a threshold level,
3) The effects of dieldrin on tumour formation are essentially irreversible and cumulative regardless of the time schedule of application.

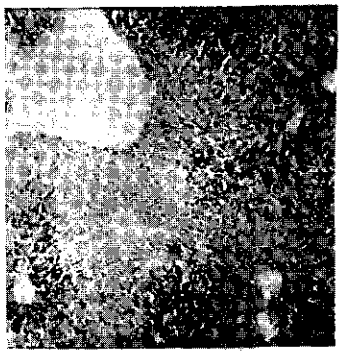
The fact that even limited exposures of CF-1 mice to dieldrin lead to an increased liver tumour risk clearly contrasts with the experimental observations obtained with skin tumour promotors. In a classical two-stage model experiment using 2-acetylaminofluorene as the initiator of hepatocarcinogenesis and phenobarbital as the promotor, Peraino et al. (1977) also found evidence for the irreversibility of liver tumour promotion. Pro-gressive increases in the duration of phenobarbital treatment shortened the tumour-induction periods and increased overall tumour incidence levels. These results demonstrate that liver tumour promotion may not be regarded as a reversible process.

These consideration are critically important in the assessment of the health effects associated with human exposure to tumour promoting agents. Moreover, the lack of evidence for a threshold level of dieldrin mediated liver tumour promotion further emphasizes the need for a re-evaluation of the health policy and risks associated with liver tumour promoters. In this context it is important to note that the effects of a liver tumour promoter will be more severe in populations exposed to high levels of initiators of hepatocarci-nogenesis. These populations may be found, for example, in the developing countries, where exposure to aflatoxin B1 and chronic hepatitis B (both initiators of hepatocarcinogenesis) affect the health of a large proportion of the population.

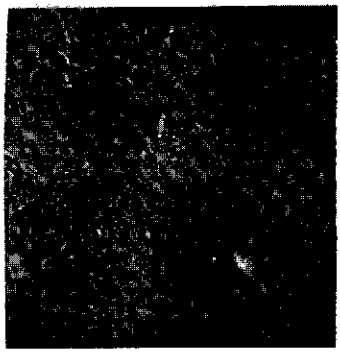
The tumour-enhancing effects of microsomal enzyme inducers, such as dieldrin, have been demonstrated not to be associated with any genotoxic action. The induction of liver enlargement and microsomal enzyme systems suggests that dieldrin mediates its action by increasing the functional pressure on hepatocytes. Although there may be more than one receptor for dieldrin it can be demonstrated by the use of specific P-450 isoenzyme antibodies (Fig. 1) that dieldrin mainly induces the P-450 d isoenzyme (van Ravenzwaay, unpublished results).

The results of the immuno-histochemal staining of liver sections from CF-1 mice exposed to 10 ppm dieldrin in the diet, suggest that dieldrin induces isoenzymes of the isosafrole type (see Table IV, Chapter 2.4.1).

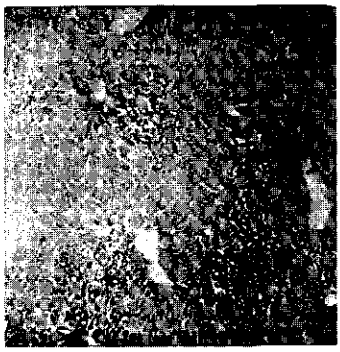
The tumour enhancing effects of increased functional commitment in hepatocytes have gained further support from experiments conducted by Rehm et al. (1985). In their experiment performed with NMRI mice it was observed that food restriction, which reduces the functional commitment in hepatocytes, delayed the development of (spontaneous) tumours, but had no influence on overall tumour incidence.



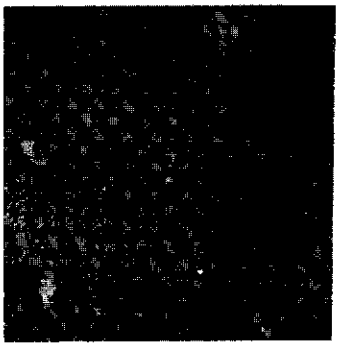
P-450 b Control



P-450 b 10 ppm dieldrin

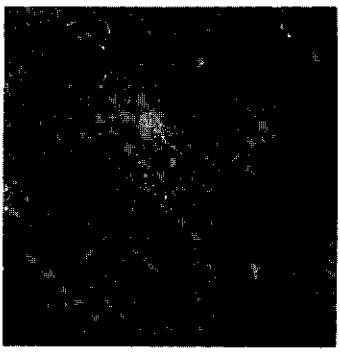


P-450 c Control

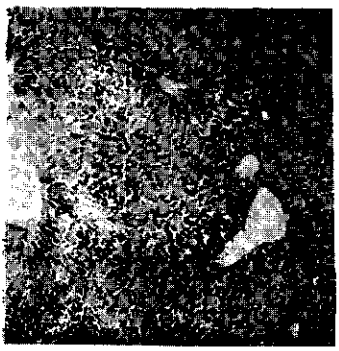


P-450 c 10 ppm dieldrin

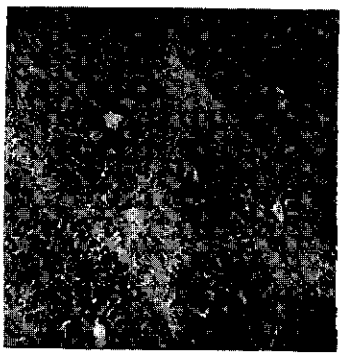
P-450 d Control



P-450 d 10 ppm dieldrin



P-450 f Control



P-450 f 10 ppm dieldrin

Figure 1. Immunohistochemical staining of different P-450 isoenzymes in control and 10 ppm dieldrin treated CF-1 mouse liver

A similar relationship between the level of functional pressure and nuclear polyploidization has been observed: induction of microsomal enzymes with phenobarbital enhances polyploidization (Böhm and Noltemeyer, 1981), whereas food restriction decreases the level of polyploidization (Enesco and Samborsky, 1983). The results of the determination of nuclear polyploidization in the livers of CF-1 mice presented in this thesis, demonstrate that nuclear polyploidization, as expressed by the proportion of octaploid (8c) nuclei, increases linearly with age. In addition, a dose-dependent enhancement of polyploidization was observed within 1,85 months after commencement of dieldrin treatment. Most importantly it was established that liver tumour formation was associated with a constant degree of polyploidization for all treatment groups, including controls. On the premiss that polyploidization reflects the ageing process, it may be concluded that dieldrin advances the biological age of CF-1 mouse liver. It is conceivable that the increased functional pressure exerted by dieldrin on hepatocytes may accelerate the ageing process.

The influence of dieldrin on the expression of isoenzymes in the liver can be divided into two components. During the initial phases of treatment a shift towards fetal types of pyruvate kinase and lactic dehydrogenase was observed. This shift was demonstrated to be reversible and may be regarded as an adaptation of the liver to dieldrin exposure. In contrast, it was observed that dieldrin dose-dependently advances the age dependent shift in alanine amino transferase in "steady-state" situations. These findings emphasizes the importance of accelerated biological ageing as a mechanism in dieldrin-mediated liver tumour formation.

Further studies on the role of nuclear polyploidization on hepatocarcinogenesis in CF-1 mice revealed a marked decrease in the tetraploid (4c) : diploid (2c) ratio, approximately 4 months before the median liver tumour induction period, in all treatment groups including controls. The

observation that the decrease in the 4c:2c ratio which occurred in all treatment groups a few months before liver tumour formation, and the fact that such a decrease has previously been found to be associated with hepatocarcino-genesis (Styles et al., 1985) suggested a possible relationship between this event and the subsequent liver tumour formation. Two mechanisms are proposed to account for such a relationship:

1) A reduction in the 4c:2c ratio could result from an increased turnover of tetraploid cells. Since liver weight has been observed not to change during the entire experiment, the lost tetraploid cells are likely to be replaced by diploid ones, resulting in a proliferative stimulus on this cell population.

2) Amitotic division of tetraploid nuclei would also result in a reduction of the 4c:2c ratio without a change in liver weight. Chromosomal re-arrangements during this event could turn heterozygous mutations homozygous.

Both hypotheses imply that the diploid population is the origin of the tumour. The results from the determination of polyploidization in liver tumours confirmed that these tumour originate from a diploid population. An artificial reduction of the 4c:2c ratio (induced by disconti-nuation of dieldrin treatment) performed with mice previously labelled with ^{3}H-thymidine resulted in a slight increase of labelled diploid nuclei suggesting that some amitotic divisions had occurred. Interestingly, the increased levels of ^{3}H-thymidine in polyploid nuclei observed during dieldrin treatment (indicating DNA synthesis for polyploidization) had returned to control level, 3 weeks after discontinuation of treatment. These findings suggest that the reversibility of liver enlargement (and polyploidization) may be attributed to the elimination of the excess polyploid cells induced during liver enlargement. This "memory" effect greatly increases the chance that heterozygous mutations, duplicated

during polyploidization, will turn homozygous as a result of amitotic divisions. The irreversible enhancement of liver tumour formation, even after a very short exposure to dieldrin, also seems to favour a genetic mechanism. However, that chronic and limited exposure of CF-1 mice to dieldrin enhances liver tumour formation following c t kinetics suggests a mechanism involving the cumulative effects of dieldrin exposure, which are associated with the accelerated expression of the intrinsic neoplastic potential in CF-1 mouse liver.

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4. Summary

A variety of xenobiotic compounds is known to induce characteristic changes in the livers of laboratory animals. These changes include enlargement of the liver, usually as a result of cell enlargement (hypertrophy) or increased cell replication (hyperplasia), induction of drug-metabolizing enzymes and proliferation of the smooth endoplasmic reticulum (SER). Such changes are usually not accompanied by evidence of liver damage and thus are reversible upon withdrawal and elimination of the compound. Consequently, most authors regard this phenomenon as an adaptive response of the organ to increased functional demands.

However, chronic exposure of various strains of mice to dieldrin, phenobarbitone, DDT and the α-, β- and γ-stereoisomers of hexachlorocyclohexane (HCH, also known as benzenehexachloride, BHC) may lead to the development of liver tumours.

The tumorigenic effects of microsomal enzyme inducers in mice may result from (a) a weak carcinogenic action of the xenobiotics themselves or (b) an enhancing (promoting) action of xenobiotics on a pre-existing oncogenic factor in mouse liver. The first objective of this study was to discriminate between these two possible types.

Druckrey and his associates have established both theoretically and experimentally the dose-response characteristics of chemical carcinogens:

$$D \cdot T^n = \text{constant} \qquad (1)$$

where D = daily dose, T = the median tumour induction period and n = an exponent, always > 1.

Since the mechanisms by which enhancers or promotors of carcinogenesis operate is quite different from the one used by carcinogens, it is be conceivable that promotors also exhibit different dose-response

characteristics.

The dose-response characteristics of dieldrin-mediated enhancement of liver tumour formation in CF-1 mice were analysed, using existing tumour data from chronic feeding studies at six exposure levels of dieldrin (a model compound for microsomal enzyme induction). It was found that the dose-response relationship can be expressed as:

$$(d_0 + \delta x) \cdot t = \text{constant} \qquad (2)$$

where d_0 stands for the background dose equivalent required for the induction of spontaneous liver tumours, δx represents the actual dieldrin dose (ppm in the diet) and t the median tumour induction period in the respective treatment groups. It was also established that the dose-response characteristics of limited dieldrin exposures and those of delayed exposure were consistent with equation (2), which is a Druckrey relation where n = 1.

From these findings it is concluded that dieldrin interacts reversibly with its receptors, resulting in an acceleration of tumour formation (which is essentially irreversible); dieldrin may thus be regarded as a tumour promotor. The validity of equation (2) for both chronic and limited dieldrin exposure indicates that (a) the velocity of liver tumour development is proportional to the daily dose level (δx), (b) the total tumorigenic dose is constant across all doses, (c) the effects of dieldrin on the neoplastic process in mouse liver are essentially irreversible and cumulative, and (d) there is no evidence for a threshold level.

Tumour formation is a dose- and time-dependent process. The induction of liver enlargement, microsomal enzyme systems and proliferation of the smooth endoplasmic reticulum by dieldrin are only dose-dependent. In contrast, polyploidization is dose- and time-dependent. To establish a

possible link between microsomal enzyme induction, nuclear polyploidization and liver tumour formation, nuclear polyploidization in livers of CF-1 mice was studied at five different dieldrin dose levels from 1.85 months up to tumour development. Nuclear polyploidization, expressed in the proportion of octaploid (8c) nuclei, was found to be characterized by a linear increase with age in untreated control CF-1 mice. Dieldrin treatment induced a dose-dependent increase in the proportion of 8c-nuclei in the initial phases of treatment. In "steady-state" situations nuclear polyploidization (as expressed by the percentage of 8c-nuclei) was maintained on a dose-dependent, higher level, and the percentage was was observed to increase with age, the velocity of which was the same as in untreated controls. Tumour formation was found to be associated with a constant degree of nuclear polyploidization in all treatment groups including controls. The observed quantitative link between nuclear polyploidization and tumour formation leads to the question whether or not a causal relationship between the two exists. Assuming that polyploidization reflects the ageing process, the data suggest that liver tumour formation is imminent at a constant biological age and that dieldrin could operate by advancing the biological age of CF-1 mouse liver.

Further support for this hypothesis was obtained from the determination of cytoplasmic alanine amino transferase (AAT) isoenzymes. The expression of the isoenzyme decreases with age in untreated control CF-1 mice. Dieldrin treatment was found to enhance (accelerate) this process in a dose-dependent manner.

Although the nature of the development of "spontaneous" liver tumours in CF-1 mice remains unknown, the decrease in the tetraploid(4c)-diploid (2c) ratio of liver nuclei, observed in the study of polyploidization, may be related to tumour formation. The decrease was observed in all

treatment groups, including controls, and its onset was dose-dependently advanced by dieldrin treatment, occurring approximately 4 months before the median liver tumour induction period in all cases. Two mechanisms are proposed that may explain the tumorigenic features of a decrease in the 4c-2c ratio.

1. Tetraploid cells could be more sensitive to accumulative toxic stress. Thus, their turnover may be increased. To replace one tetraploid cell a diploid cell has to divide twice; the loss of tetraploid cells would therefore result in a proliferative response of the diploid population (resulting tumour formation).
2. A reduction in the 4c-2c ratio could be induced by the occurrence of amitotic nuclear divisions in the tetraploid cells. Evidence for this possibility was obtained from experiments with ^{3}H-thymidine-labelled nuclei. Amitotic nuclear divisions could give rise to chromosomal rearrangements, resulting in the expression of the intrinsic neoplastic potential of CF-1 mouse liver.

Both hypotheses imply that the diploid population is the source of liver tumours. The determination of nuclear polyploidization in liver tumours confirmed that these tumours originate from the diploid liver cell population.

5 SAMENVATTING

Vele lichaamsvreemde stoffen zijn in staat karakteristieke veranderingen te induceren in de levers van proefdieren. Deze veranderingen zijn in het algemeen als volgt samen te vatten: leververgroting als gevolg van een kombinatie van cel vergroting (hypertrophie) en cel vermeerdering (hyperplasie), inductie van microsomale enzymen voor de biotransfomatie van lichaamsvreemde stoffen, en proliferatie van het gladde endoplasmatische reticulum. Gewoonlijk hebben deze veranderingen geen lever beschadeging tot gevolg en zijn reversibel na stopzetting van de behandeling. Deze veranderingen worden daarom door velen als een aanpassing van de lever aan verhoogde functionele belastingen beschouwd. Chronische behandeling van muizen met verschillende lichaamsvreemde stoffen zoals DDT, dieldrin, phenobarbital en een aantal hexachloorcyclohexaan (HCH) isomeren, kunnen echter de ontwikkeling van lever-tumoren tot gevolg hebben.

De kankerverwekkende eigenschappen van microsomale enzym inductoren kunnen het gevolg zijn van:

1) een zwak carcinogeen effekt van de lichaamsvreemde stof zelf, of,
2) een versterkend effekt van de lichaamsvreemde stof op een reeds voorhande zijnd oncogeen potentiaal, waardoor dit tot expressie komt.

Het eerste doel van het in dit proefschrift beschreven onderzoek was een mogelijkheid te vinden om te onderscheiden tussen de hierboven genoemde werkings typen. Druckrey en zijn medewerkers toonden, meer dan 25 jaar geleden, aan dat de dosis-tijd-werkingsrelaties van chemische carcinogenen beschreven kunnen worden door:

$$D \cdot T^n = \text{constant} \qquad (1)$$

waarin D = dagelijkse dosis, T = de gemiddelde latentie tijd voor tumor ont-

wikkeling, en n = een exponent, altijd >1. De resultaten van vele experimenten hebben de validiteit van deze formule bevestigd. Aangezien het werkingsmechanisme van versterkers of promotoren van het carcinogene proces verschilt met de werking van volledige carcinogenen, is het waarschijnlijk dat promotoren een andere dosis-werkingsrelatie zullen vertonen.

De dosis-werkingsrelatie van de door dieldrin (een typische microsomale enzym inductor) veroorzaakte leverkanker ontwikkeling in CF-1 muizen, kan wiskundig als volgt beschreven worden:

$$(d_0 + \delta_x) \cdot t = \text{constant} \qquad (2)$$

waarin d_0 = de dosis equivalenten (uitgedrukt in ppm dieldrin in de voeding) die het ontstaan van "spontane" levertumoren in de controle groepen moeten verklaren, δ_x = de werkelijke dieldrin dosis (ppm in de voeding) en t = de gemiddelde latentie tijd voor tumor ontwikkeling in de verschillende behandelings groepen.

Bovendien bleek dat zowel de dosis-werkingsrelatie voor een beperkte (niet chronische) dieldrin behandeling als ook die voor een dieldrin behandeling na een behandelings vrije periode (60 weken) in overeenstemming waren met vergelijking (2). Deze vergelijking is een Druckrey relatie (zie vgl. 1), waarbij de exponent n = 1 (bij carcinogenen: n altijd >1).

Uit deze resultaten kan geconcludeerd worden dat dieldrin een reversibele interactie aangaat met cellulaire bestanddelen, waardoor het tumorigene proces versneld wordt (een versnelling van de carcinogenese kan niet anders dan irreversibel zijn). Dientengevolge kan dieldrin als een tumor promotor beschouwd worden.

Uit vergelijking (2) kan worden afgeleid dat, bij behandeling van CF-1 muizen met dieldrin:

1) De snelheid van leverkanker ontwikkeling proportioneel is aan de som van

de dagelijkse dieldrin dosis en de dieldrin equivalenten voor de levertumor ontwikkeling in de controle groepen.

2) De totale tumorigen dosis, gegeven tot het optreden van lever kanker, is constant voor alle behandelings groepen

3) De gevolgen van dieldrin behandeling op de tumor ontwikkeling zijn irreversibel en cumulatief.

4) Er zijn geen aanwijzingen gevonden die op het bestaan van een drempelwaarde duiden.

Kanker ontwikkeling is een dosis- en tijds-afhankelijk proces. De inductie van lever vergroting, de proliferatie van het gladde endoplasmatische reticulum en de inductie van microsomale enzymen, zijn allen dosis afhankelijk. Polyploidisering echter, is een proces dat, net als kanker ontwikkeling, zowel van de dosis als ook van de tijd afhankelijk is.

Om een mogelijk oorzakelijk verband tussen microsomale enzym inductie -- polyploidisering -- leverkanker vast te kunnen stellen werd de polyploidiserings graad van hepatocyten bepaald bij 5 verschillende dieldrin behandeling niveaux vanaf 8 weken behandeling tot tumor ontwikkeling. Polyploidisering, uitgedrukt in het percentage octaploide (8c) kernen, in onbehandelde CF-1 muizen werd gekenmerkt door een lineaire toename met de tijd. Dieldrin behandeling resulteerde in een dosis-afhankelijke toename van het percentage 8c kernen in het begin van de behandelings periode. Na deze initiele toename steeg het percentage 8c kernen met dezelfde (lineaire) tijds-afhankelijkheid als in de controle groep. Levertumor ontwikkeling bleek te zijn verbonden met een constante polyploidiserings graad (ca. 21% 8c kernen) in alle behandelings groepen.

De gevonde quantitatieve relatie tussen polyploidisering en levertumor ontwikkeling riep de vraag op of een causaal verband tussen deze waarnemingen

bestond. Polyploidisering is een proces dat veroudering weerspiegeld; de toename van de polyploidisering onder invloed van dieldrin behandeling zou dus als een versnelling van het verouderings proces opgevat kunnen worden. De resultaten van de isoenzym bepaling van het cytoplasmatische alanine-amino transferase (AAT) ondersteunen deze hypothese. De expressie van het A-AAT isoenzym bleek in onbehandelde CF-1 muizen af te nemen bij toenemende leeftijd. Behandeling met dieldrin had een dosis-afhankelijke versnelling van dit proces tot gevolg.

Alhoewel de oorzaak van de levertumor ontwikkeling in CF-1 muizen nog onbekend is, zou de afname in de verhouding van tetraploide (4c) - diploide (2c) lever kernen met het ontstaan van leverkanker te maken kunnen hebben:

a) De afname in de 4c-2c verhouding vond in alle behandelings groepen plaats. b) Deze afname trad, dosis-afhankelijk, eerder op bij hogere doseringen, en wel zo dat het begin van dit proces ongeveer 4 maanden voor de gemiddelde tumor latentie tijd lag. Twee verschillende hypothesen zouden een verklaring kunnen geven voor een causaal verband tussen de afname in de verhouding van 4c-2c lever kernen en tumor ontwikkeling.

1) Mogelijk zijn tertaploide kernen gevoeliger voor (cumulatieve) toxische belasting. Hierdoor zouden deze, onder invloed van dieldrin belasting, sneller afsterven. Om een tetraploide cel te vervangen moet een diploide cel zich twee maal delen . De afname in het aantal tetraploide kernen zou dus, bij onveranderde lever grootte en gewicht, een sterke delingsactiviteit bij de diploide cellen tot gevolg kunnen hebben (met daaruit resulterende activering van het oncogene potentiaal in de lever van CF-1 muizen).
2) Een afname in de verhouding van 4c-2c lever kernen zou ook verklaard kunnen worden door het optreden van amitotische delingen. Aanwijzingen dat

een dergelijk proces daadwerkelijk op kan treden in de lever van CF-1 muizen zijn verkregen met ^{3}H-thymidine gemarkeerde kernen. Amitotische delingen kunnen genetische herverdeling tot gevolg hebben waardoor recessieve kenmerken tot expressie kunnen komen.

De hier voorgestelde hypothesen impliceren dat leverkanker zou ontstaan uit dioploide cellen. Uit bepalingen van de polyploidiserings graad van lever tumoren bleek dat deze inderdaad ontstaan waren uit een diploide celpopulatie.

CURRICULUM VITAE

Bennard van Ravenzwaay werd geboren te Hilversum op 2 december 1960, en behaalde in 1979 het Atheneum β diploma aan Het Nieuwe Lyceum te Hilversum. In datzelfde jaar werd begonnen met de studie millieu-hygiene aan de Landbouwhogeschool (thans Landbouwuniversiteit) te Wageningen, waar hij in 1985 het ingenieurs diploma behaalde. De hoofdvakken tijdens zijn studie waren dierfysiologie (prof. dr. Schoonhoven) en toxicologie (prof. dr. Koeman) en als bijvak waterzuivering (prof. dr. Lyklema).

In de laatste fase van de studie werd een stage periode aan het Deutsche Krebsforschungszentrum te Heidelberg in West Duitsland gelopen. Direkt in aansluiting op deze stage begon hij in het Deutsche Krebsforschungszentrum in het Institut für Biochemie, Abteilung Molekulare Toxikologie bij prof. dr. Kunz het promotie onderzoek, dat uitgevoerd werd in het kader van een samenwerking tussen deze vakgroep en de vakgroep toxicologie van de Landbouwuniversiteit. De resultaten van dit onderzoek zijn beschreven in dit proefschrift.