A Survey of the Embryotoxic Effects of TCDD in Mammalian Species

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Introduction

Only limited data are presently available on the embryotoxic effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). This is true for the number of animals used per experimental group, especially in the higher dose range, as well as for the number of different species of experimental animals tested. There has been an understandable reluctance to investigate these highly toxic compounds, because of the hazards of contaminating animal quarters and the risk to personnel. Nevertheless, we feel that the data available allow a rough estimate of the dangers that may be encountered with exposure to such dioxins during pregnancy.

The possibility that TCDD might have certain types of embryotoxic effects was realized for the first time when Courtney et al. (*1*) reported their data on the teratogenic effect of 2,4,5-T. Substances like TCDD have been known for some time (*2–4*) to be contaminants of chlorinated phenols and derivatives of such compounds and they were thought to be responsible for an intoxication called chloracne (*5*). Some commercial samples of 2,4,5-T apparently were rather highly contaminated with these extremely toxic substances.

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In this paper we evaluate exclusively data which have been obtained using pure TCDD. No attempt is made to analyze the rather extensive literature on results obtained with 2,4,5-T samples contaminated to various degrees with TCDD or similar compounds.

Some Special Aspects of Embryotoxic Effects Induced by TCDD

Some General Aspects of Embryotoxic Action

Considerable confusion has been caused by many previous investigators with the nomenclature used in the field of prenatal toxicology. Unfortunately, the term used to specify certain experimental data in this field may suggest a special mode of action (*6*) and, more important, may trigger conclusions on a possible hazard to be expected during human embryonic development.

We, therefore, wish to suggest a system of nomenclature used by our group which is in accordance with the terms used in toxicological research and which at the same time takes into account the special situation of prenatal development.

The most comprehensive term is embryotoxic (fetotoxic) effect. A special situation of an embryotoxic effect may be characterized, e.g., a teratogenic effect, an embryolethal (fetolethal) effect, or a retardation (retarded growth) etc.

We feel that the following definitions prove to be convenient. By embryotoxic (fetotoxic) effects we denote all transient or

permanent toxic effects induced in an embryo or fetus, regardless of the mechanism of action. By embryolethal (fetolethal) effect or embryomortality (or fetomortality) we denote prenatal mortality at any stage of embryonic or fetal development (may be referred to as $LD_5 - LD_{95}$). This term is certainly not identical with embryotoxic but refers to a special event that may occur in the course of an embryotoxic action.

We define a teratogenic effect as an abnormality originating from an impairment of an event typical for embryonic or fetal development (induction process, differentiation). The abnormality should be largely irreversible. It may be obvious by macroscopic appearance or "hidden" (micromorphological defect or inborn error of metabolism) or result in a mental abnormality.

Some examples of embryotoxic, not teratogenic effects are: general retardation of embryonic (or fetal) growth, retarded occurrence of certain ossification centers (reversible), fetal intestinal hemorrhages (without secondary effects). Representing a teratogenic effect would be an irreversible involution of lymphatic tissues (with consequences in postnatal life) or a severe mental defect resulting from an impairment of prenatal brain development.

Some Special Aspects Connected with the Action of TCDD

A brief analysis of the effects of TCDD on prenatal development may suggest that TCDD is a surprisingly specific teratogen, interfering only with a few special developmental processes. An increased frequency of cleft palate and kidney abnormalities of a special type are the only teratogenic effects which have been observed. Interestingly enough, it has not been possible so far to induce limb or head abnormalities with this agent, despite the fact that the drug was given at the "critical period" for the induction of such malformations. Larger experimental series over a sufficient dose range are necessary to permit conclusions on the specificity of the teratogenic action of TCDD, however.

TCDD is certainly not a teratogenic agent exclusively, since an increased fetomortality results if the drug is given at a high enough dose over a long enough time interval, although with such a dose schedule no obvious symptoms of a maternal toxicity become evident. Apparently the higher doses lead to a more general toxic effect on the embryonic cells, but an effect on the placenta or maternal tissues cannot be excluded at the moment.

Some of the toxic signs which can be demonstrated in the fetal tissue should not be referred to as teratogenic. This includes the intestinal hemorrhages (without permanent secondary changes), a fatty infiltration (Fig. 1) of fetal livers (*7*) (to our knowledge not reported before with any embryotoxic agent), as well as subcutaneous edema and delayed ossification. Most of these symptoms are reversible and they are certainly not the result of a typical interference with developmental processes. It is interesting that most of these symptoms can also be demonstrated in tissues of adult organisms under the toxic action of TCDD.

In adult mice a pronounced involution of some lymphatic tissues can be demonstrated as an early toxic sign, together with a loss of weight (*8*), predominantly in a drastic reduction of the size of the thymus, the spleen, and lymph nodes (*9*). A similar interference has been observed in our group with the development of these systems during fetal development in the presence of TCDD (*10*). This impaired development of lymphatic organs results in a typical postnatal insufficiency and in a pronounced reduction of the chances of postnatal survival.

Although these symptoms very much resemble changes produced in the adult organism by TCDD (*9*), we would consider these embryotoxic effects as teratogenic, since a typical developmental process is altered and the defect can be demonstrated long enough to handicap the newborn.

From all our studies we have seen no indication that the general growth of the fetal mice showing a teratogenic effect was affected to a significant degree.

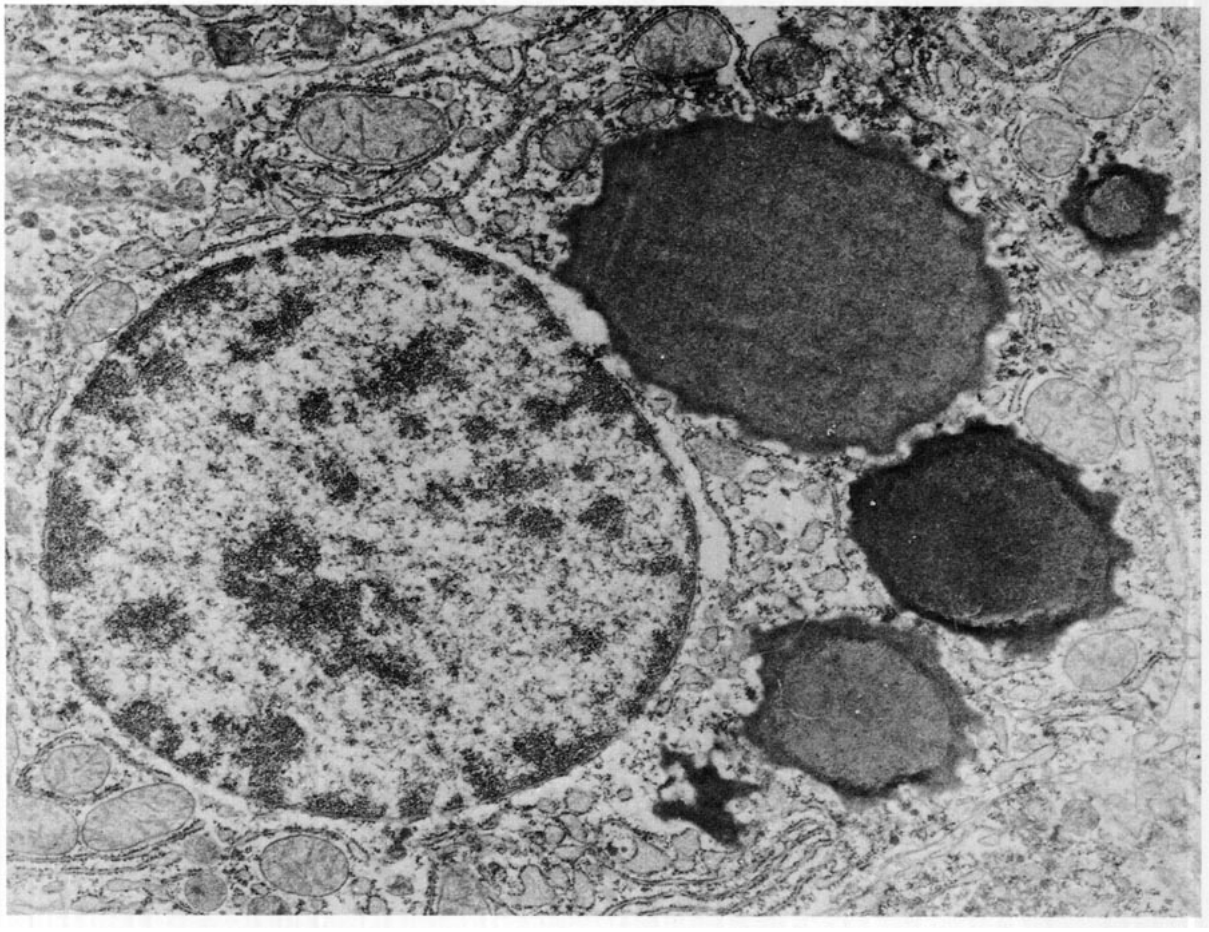

FIGURE 1. Example of an electron microscopic examination of fetal rat liver (day 18 of gestation). TCDD (15 μg/kg) was given once on day 17 of pregnancy. Using TCDD it has been possible to produce a fatty infiltration of the developing fetal liver as early as on day 14 of gestation.

We feel that the sort of embryotoxic effects induced by TCDD are very interesting from a theoretical point of view and to some extent unique. Therefore, they justify a further, closer analysis.

Results and Discussion

First indications of embryotoxic effects of TCDD were reported in 1970 by Sparschu et al. (*11*, *12*) from experiments with rats. Further data on embryotoxic effects then became available from reports of Courtney and Moore (*13*) and from our group (*14*) (see Table 1).

Three major effects were noted in these studies: intestinal hemorrhages in rat fetuses and an increased frequency of cleft palate and kidney abnormalities in mouse fetuses. Teratological studies with rats have apparently only been performed using repeated doses of TCDD while with mice effects produced by single as well as by repeated doses of this drug have been reported.

We shall try to evaluate the data from teratological studies available and to supplement them with unpublished data obtained in our laboratory. The following aspects will be discussed: (1) dose–response relationships after repeated doses of TCDD given to rats and mice; (2) dose–response relationships after single doses of TCDD given to mice and

Table 1. Evaluation of the published teratogenic (embryotoxic) effects induced by TCDD in rats and mice.[a]

Species	Strain	Teratogenic effect (system)	Dose μg/kg Minimal tested	Dose μg/kg ≈ED_{50}[b]	Time TCDD given, days	Route	Reference
Rat		Intestinal hemorrhage	0.125	= 0.5 ?		Oral	Sparschu et al. (*12*)
	CD	Kidney abnormality	0.5	> 1	6–15	SC	Courtney and Moore (*13*)
Mouse	CD–I	CP	1 ? 3	> 3	6–15	SC	"
		Kidney abnormality	1	1–3	6–15	SC	"
	DBA/2J	CP	3	> 3	6–15	SC	"
		Kidney abnormality	3	> 3	6–15	SC	"
	C57Bl/6J	CP	3	> 3	6–15	SC	"
		Kidney abnormality	3	< 3	6–15	SC	"
	NMRI	CP	3	6.5	6–15	Oral	Neubert and Dillmann (*14*)
			9	< 9	9–13	Oral	"
			15	40	13	Oral	Neubert et al. (this paper)
			5	15	11	Oral	" "

[a] The smallest dose with which a significant teratogenic effect has been produced is indicated. Since sometimes only one dose level was tested this does not necessarily give the smallest dose from which a teratogenic effect could result. Routes were both oral and subcutaneous (SC). An attempt was also made to estimate the ED_{50} from the few data available.

[b] ED_{50}: dose required to produce effect in 50% of animals.

evaluation of phase specificity; (3) embryotoxic effects observed after application of TCDD together with other teratogens.

Dose–Response Relationship of Teratogenic Effects and Fetomortality After Repeated Doses of TCDD to Rats or Mice

The first striking result which is immediately apparent is the extremely low dose of TCDD which is able to induce teratogenic and fetolethal effects in mice and rats. Although these species must be considered to be comparatively insensitive during the adult status towards the toxic action of this compound, repeated or even single doses of as little as 1–10 μg/kg are capable of reproducibly triggering malformations of certain types. This is by far the smallest effective dose of any teratogen known today.

Interestingly, of the series of chlorinated dibenzodioxins, the tetrachloro derivative apparently is the most active one. The hexachloro derivative also seems to show some embryotoxic activity, while the dichloro and the octachloro derivatives have been reported (*15*) to be nonembryotoxic. The few data available suggest that embryotoxic activity is a property of those compounds having a pronounced chloracne potency (*15*).

Teratogenic effects induced by TCDD—Two major types of malformation have been reported so far in fetuses of rats or mice treated with TCDD during pregnancy: cleft palate—so far reported only in mice—and kidney abnormalities of a certain type, observed in both species after single or repeated doses of TCDD (Table 2).

Table 2. Embryotoxic effects induced by TCDD.[a,b]

Species	Abnormality	Dose producing ~50% effect, μg/kg [c]
Rat	Intestinal hemorrhage	0.5 ?
	Kidney abnormalities	1–2 ?
	Lethal	1–2 ?
Mouse	Cleft palate	6
	Kidney abnormalities	1–3
	Lethal	7

[a] TCDD was given on days 6–15 of pregnancy.
[b] Literature data (*11, 13, 14*).
[c] ? = no exact data available from the literature. Doses assumed to be in the range indicated.

Furthermore, an involution of fetal thymus and spleen and other lymphatic organs can be observed (*10*) which affects the survival after birth. Intestinal hemorrhage and fatty infiltration of the fetal liver do not represent teratogenic effects, as discussed above. Within the adult organism similar symptoms can be noticed.

We are convinced that looking more closely at fetuses affected by TCDD will reveal more abnormalities; this has been the case with every drug effect evaluated carefully enough with the modern biochemical and micromorphological methods available.

Other than the reports on teratogenic and embryotoxic effects produced by TCDD in mice and rats with documented experimental data we found only a few references which suggest that embryotoxic effects may also occur in other animal species after a treatment with TCDD. Although we did not find verifiable data, TCDD apparently can also produce embryotoxic effects in hamsters. Eye abnormalities and reduction of mean fetal weight—neither of which is typical of symptoms seen in rats or mice after treatment with TCDD—as well as gastrointestinal hemorrhages and increased prenatal mortality are mentioned after doses of TCDD in the μg/kg range for 5 days (*16*).

Unfortunately, we have no knowledge of teratological experiments performed with the highly TCDD-sensitive species guinea pig and rabbit. It would be important to know whether the comparatively high toxicity seen in adult animals of these species is also paralleled by a high degree of sensitivity of the embryos or fetuses when compared with that of rats and mice.

Fetomortality induced by TCDD—Although the teratogenic effect induced by TCDD seems to be rather specific because the development of only a limited number of special organ systems is found to be impaired, TCDD when given in multiple doses does not represent an exclusively teratogenic agent. The occurrence of developmental abnormalities in chronic experiments is almost paralleled in a dose-response curve by the fetomortality (Fig. 2). However, as in many teratological experiments, there is no obligatory connection of these two parameters. In mice a high cleft palate frequency can be obtained without any apparent fetomortality (Fig. 3), just by reducing the time of treatment from 10 days (day 6–15) to 5 days (day 9–13 of gestation).

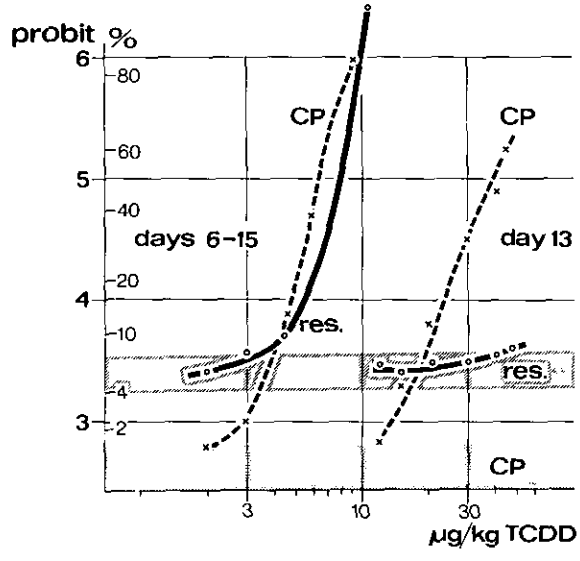

FIGURE 2. Comparison of the teratogenic effect and the degree of fetomortality induced by TCDD. The effect of a single dose given on day 13 of pregnancy is compared with that resulting after repeated doses given during days 6–15 of pregnancy. The points are derived from the evaluation of the total fetuses per treated group of mice (at least 12 litters per dose). TCDD was given in rape-seed oil by stomach tube once a day (1 P.M.). Cleft palate frequency is evaluated in this experiment as percent of the viable fetuses. Effect (probit scale) is plotted against dose (log scale). The control values represent 2000 fetuses from mice treated with rape-seed oil for 10 days.

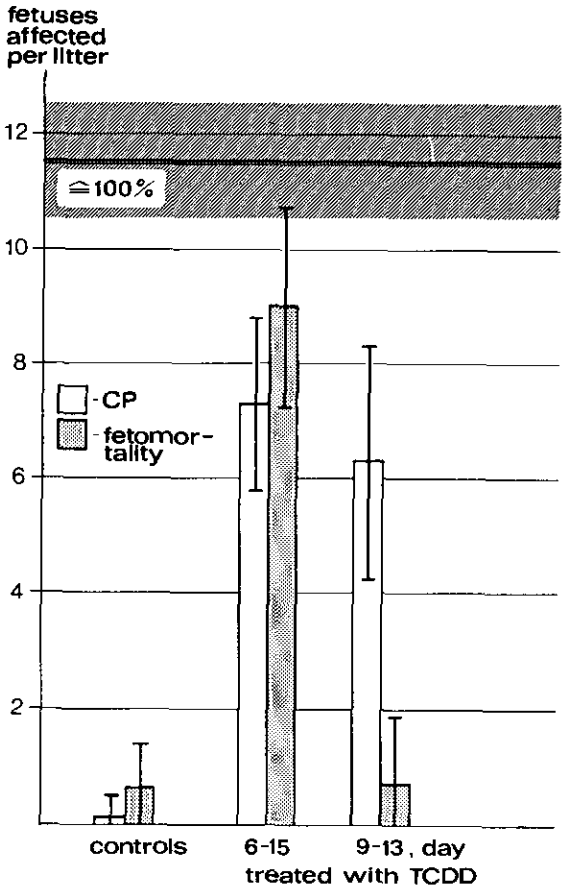

FIGURE 3. Comparison of teratogenic effect and fetomortality produced by TCDD. Ten pregnant mice each were treated with 9 μg/kg TCDD orally during the time interval indicated. The numbers of fetuses affected are given ($\bar{M}$ ± S.D.) per litter. Average number of implantation sites per litter was 11.5 (= 100%). Controls received rapeseed oil only. CP denotes cleft palate frequency.

Differences among strains in the degree of teratogenic and fetolethal effects induced by TCDD in mice—Information on differences in the susceptibility towards TCDD of different strains of experimental animals are so far available only for mice, mainly through the data of Courtney and Moore (*13*). Tables 3 and 4 summarize these data, supplemented by information obtained with NMRI mice in our laboratory (*14*). For a better comparison we have recalculated some of the data. Since for half of the strains data are only available at one dose, the comparison is made for the effect produced by 3 μg/kg TCDD given during days 6–15 of gestation.

As can be seen from Table 3, with this scheme of treatment a noticible but small fetomortality occurs only with the CD–1 mice. With the same scheme of treatment a significant increase in the cleft palate frequency over that of the controls can be observed with all of the four strains tested. While the incidence of this type of teratogenic effect is roughly the same with three of the four strains (3–4%), the C57Bl mice are clearly more susceptible to the teratogen. Further data of Courtney and Moore (*13*) lead to the conclusion that the special sensitivity of the C57Bl mice also holds for the induction of kidney abnormalities. At the moment no clue is available for the cause of this higher sensitivity. An increased susceptibility of the target tissue or alternatively a special rate of drug metabolism in this strain, leading to a higher concentration of the effective drug in the fetus, may be responsible for this special sensitivity. Further data are needed to distinguish between these possibilities.

Dose–Response Relationship of Teratogenic Effects and Fetomortality in Mice after a Single Dose of TCDD (Phase Specificity)

We have started some systematic studies to clarify the question whether the teratogenic effects may be induced by single doses of TCDD—and by what doses—and what appears to be the most sensitive interval of fetal development. The induction of cleft palates was chosen as a criterion in these studies, but other types of malformation (kidney abnormalities) may also be evaluated from these experimental series.

The highest degree of malformations can be produced (*14*) when the drug is given to the mice on day 11 of pregnancy. Although a significant increase in the cleft palate frequency over that of the controls can also be induced by giving the teratogen on days 10 or 12 of gestation, the effect obtained on these days is only about half of that produced on day 11 for a given strain.

Table 3. Fetomortality induced by TCDD in different strains of mice.[a]

Mouse strain	Avg. fetomortality, %/litter[b]	
	TCDD	Controls
CD-1[c]	12.4 (144)	8.6
NMRI[d]	5.3 (110)	4.8
DBA[c]	27.0 (103)	26.1
C57Bl[c]	5.3 (49)	10.8

[a] TCDD, 3 μg/kg, was given daily on days 6–15 of pregnancy.
[b] Values in parentheses denote percentage when compared with the corresponding controls receiving the vehicle only. In none of the strains tested was a pronounced fetomortality observed.
[c] TCDD given subcutaneously (SC) in DMSO; data of Courtney and Moore (*13*).
[d] TCDD given orally in oil; data of Neubert and Dillmann (*14*).

When TCDD is given on day 13 of gestation, an effect of only about one third of that seen on day 11 is produced. It should be mentioned that in all of these experiments only one time per day (at noon) was checked since it was not intended to pinpoint exactly the time of the highest sensitivity. The maximum of the effect may, therefore, be somewhat higher.

In order to compare the cleft palate-inducing effect of TCDD with that seen after treatment of pregnant mice with other teratogens we have performed most of the following experiments by giving TCDD on day 13 of gestation, even though the effect with TCDD obtained on this day is not maximal. Interestingly enough, cleft palates can be produced by giving different teratogens with a phase optimum which varies considerably, indicating quite different modes of action of the various teratogens (Fig. 4).

Because of our interest in the dose–response relationship of the teratogenic effect of TCDD we compared the dose–response curves (cleft palate frequency) obtained with five cleft palate-inducing drugs (Fig. 5). The steepness of the dose–response curves varies considerably when the different drugs are

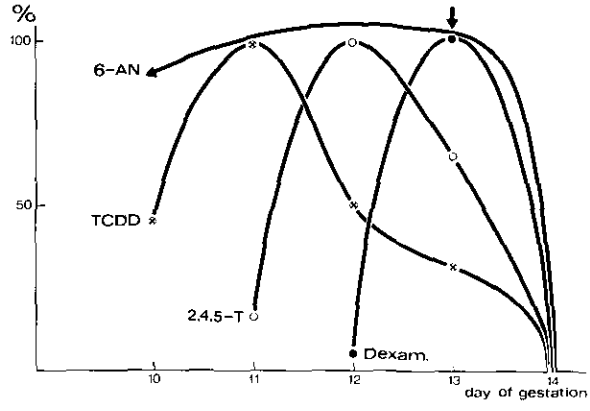

FIGURE 4. Phase specificity of cleft palate induction by various drugs. A rough survey is given on the optimal effect produced by different teratogens. The points are taken from large experimental series performed in our laboratory with NMRI mice. 100% indicates the maximum effect, not a cleft palate frequency of 100%. The arrow indicates the time at which most of our combination experiments have been performed. Not all teratogens exhibit their maximal effect at this stage of development.

Table 4. Cleft palate frequency induced by TCDD in different strains of mice.[a]

Mouse strain	TCDD			Controls	
	Fetuses evaluated	CP, %[b]	Affected litters (%)	CP, %[b]	Affected litters (%)
CD-1[c]	104	3	3/10 (30)	< 0.3	0/29 (< 3)
DBA[c]	55	4	2/9 (22)	< 1	0/23 (< 4)
NMRI[d]	271	3	7/24 (29)	0.7	10/160 (6)
C57B1[c]	58	22	5/7 (71)	< 1	0/23 (< 4)

[a] TCDD, 3 μg/kg, was given daily during days 6–15 of pregnancy (same experimental conditions as in Table 3). Strain C57Bl appears to be the most susceptible strain. All the other strains tested show about the same degree of sensitivity.
[b] % CP = percentage of cleft palates of the total fetuses examined in this group.
[c] TCDD given SC in DMSO; data of Courtney and Moore (*13*).
[d] TCDD given orally in rape seed oil, data of Neubert and Dillmann (*14*).

compared. Table 5 gives, in addition, the tan α calculated from these curves as well as the dose range required for increasing the teratogenic effect from 2% (just significantly over the controls) to 50% (probit 3 to 5).

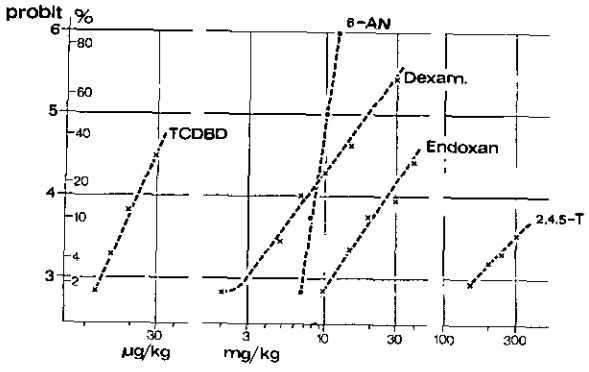

FIGURE 5. Dose-response relationship observed after single doses of different cleft palate-inducing drugs. All drugs were given on day 13 of pregnancy. TCDD and 2,4,5-T were given per stomach tube in rape-seed oil; 6-AN, dexamethasone, and cyclophosphamide were injected subcutaneously. Each point represents the frequency observed in the fetuses from at least 12 litters, at the lower dose range of at least 20 and generally about 30 litters. The data are given as percentage of the total fetuses evaluated at the dose specified. Tan α values of the dose-response curves are given in Table 5; threshold doses derived from these curves are given in Table 6.

Of the drugs tested, 6-aminonicotinamide (6-AN), a teratogen extensively studied biochemically as well as micromorphologically in our laboratory (*17–20*) shows the largest increase in cleft palate frequency with increasing doses (log dose), while 2,4,5-T shows the smallest. A dose–response curve of intermediate steepness is obtained when the effect of TCDD or of dexamethasone or cyclophosphamide (Endoxan) is plotted against the dose given. The dose required for producing a 10% effect with the various teratogens differs by more than four orders of magnitude!

Embryotoxic Effects Observed after Application of TCDD Together with Other Teratogens

Special emphasis was given to experiments

Table 5. Dose–response relationship with five cleft palate-inducing drugs.[a]

Drug	tan α of dose–response curve	Increase in dose necessary to increase effect from probit 3 to 5 (2% to 50% effect)	ED_{50} (cleft palate), mg/kg
6-AN	7.6	1.3×	10
TCDD	2.3	3×	0.040
Endoxan	1.5	5×	60[b]
Dexamethasone	1.4	6×	20
2,4,5-T	0.8	11×	2000?[b]

[a] All drugs were given to the mice as a single dose on day 13 of pregnancy, (evaluation of the data shown in Fig. 5).
[b] No 50% effect obtained; value extrapolated.

in which TCDD was combined with other teratogens. We believe that such combination studies may serve several purposes. First, very little is presently known regarding the teratogenic effect of drug combinations, despite the fact that single compounds very rarely act on an organism, either under ambient environmental conditions or during treatment. Therefore, information on the chance of potentiating effects and the possible health hazards of combined drug actions on embryonic development is urgently needed. Also, almost no information is available today on the possibility of synergistic action of two or more drugs in producing an impairment of embryonic development which cannot be induced, even by high doses, by either substance alone. Information available from studies with carcinogens suggests that the likelihood that a certain drug will induce special teratogenic effects may be greatly increased by varying nutritional factors (e.g. vitamins, protein uptake, heavy metal concentrations) or by blocking certain metabolic pathways.

When designing experiments in which the teratogenic effect of combined drug actions is to be evaluated, several possibilities have to be considered. (1) Both (or all) drugs may be given simultaneously over an extended period of time. This has the advantage that all substances act on the target tissue

at the—probably different—optimal phase of development. The disadvantage of such an experimental setup may lie in the secondary or less interesting effects of the drugs produced outside of the critical period (e.g., embryolethal effects) which could mask the teratogenic effect to be evaluated. (2) Both (or all) drugs are given only once, but at the same time. This gives more favorable conditions for evaluating the mechanism of action. However not all of the drugs may get the chance to act at the most sensitive phase. (3) Both (or all) drugs are given only once, but in sequence, each at the optimal phase of its effect. The advantages and disadvantages of this experimental design are obvious.

Each of these experimental designs has advantages when studying special problems. Each one has practical as well as theoretical implications. We feel that in the long run all the three models have to be tested in order to allow an exact elucidation of teratogenic effects of drug combinations. In the present paper we present the results of some experiments performed with TCDD combinations by use of experimental designs 1 and 2.

Cleft palate frequency induced by a combination of TCDD and 2,4,5-T given during days 6 and 15 of gestation—Studies with combinations of TCDD and 2,4,5-T were performed first, since an effect of this drug combination was of special interest. Large experimental series performed in various laboratories with 2,4,5-T preparations contaminated with dioxins to a very different extent led to such variable results that studies with a clear-cut combination of compounds were warranted.

Figure 6 shows the results of experiments in which a teratogenically active dose of 2,4,5-T (60 or 100 mg/kg) was combined with a threshold dose of TCDD or even much smaller doses.

Although we are completely aware of the fact that there is no "threshold dose" we use this term for convenience to indicate that dose (derived from a dose–response curve) which gives just no significant increase of cleft palate frequency over that of the controls, when 300–500 fetuses from treated mice and 2000 fetuses from controls are evaluated (1.5% cleft palate frequency, 0.7% seen with controls; under our experimental conditions, $\chi^2 > 3.5$).

A clear-cut effect is seen on combining 60 mg/kg 2,4,5-T with the "threshold dose" of TCDD (2 μg/kg), and a detectable potentiation is produced even with 1/10 of this

Table 6. Just nonteratogenic doses of the drugs used in our combination experiments (threshold doses).[a]

	TCDD, μg/kg		2,4,5-T mg/kg		6-AN, mg/kg	Dexamethasone, mg/kg	Endoxan, mg/kg
	Day 13	Day 6–15	Day 13	Day 6–15	Day 13	Day 13	Day 13
"Just teratogenic" dose [b]	15	3	250	40	7.5	4	15
"just nonteratogenic" dose	12	2	150	30	7	2	10
"Threshold dose" as fraction of ED_{50} [c]	1/3		1/13 [d]		7/10	1/10	1/6 [d]

[a] All doses were derived from dose–response curves, with at least 300 fetuses evaluated at the low dose levels. The data refer to single doses given on day 13 of pregnancy.

[b] The "just teratogenic" dose gives the lowest dose found to produce a significant effect ($\chi^2 > 3.5$) under our experimental conditions, 300–500 fetuses being evaluated per dose.

[c] Although we are aware of the fact that a "threshold dose" cannot be determined accurately, we use this term for the convenience of planning our combination experiments. In this paper this term is defined as the lowest dose found under our experimental conditions (300–500 fetuses) not to be able to produce an effect significantly different from the controls (2000 fetuses evaluated). For a more accurate definition this dose is also characterized as a fraction of the ED_{50}.

[d] Fetal ED_{50} extrapolated since this dose is toxic to maternal organism or fetolethal.

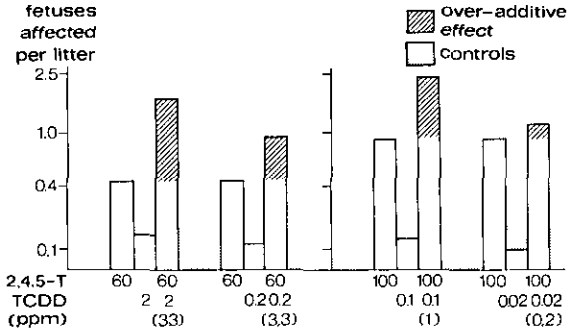

FIGURE 6. Cleft palate frequency in mice produced by a combination of TCDD and 2,4,5-T. Both drugs were given in the doses indicated (mg/kg for 2,4,5-T and μg/kg for TCDD) during the days 6–15 of pregnancy once daily by stomach tube. In each group 20 litters were evaluated. The data are given as number of fetuses per litter (*M*). The effect produced by 60 mg/kg 2,4,5-T + 2 μg/kg TCDD is highly significant, that produced by 100 mg/kg 2,4,5-T + 0.1 μg/kg TCDD just significant ($P = 0.01$).

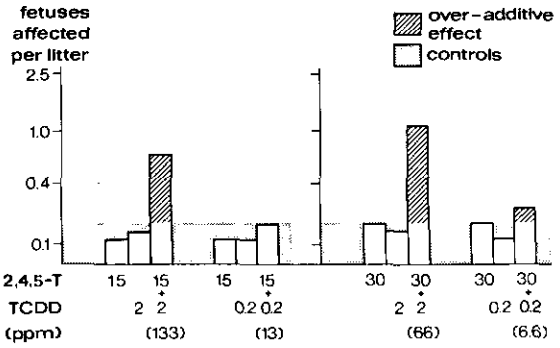

FIGURE 7. Cleft palate frequency in mice produced by a combination of TCDD and 2,4,5-T. Experimental conditions as in Fig. 6. The effects observed with both 15 mg/kg 2,4,5-T + 2 μg/kg TCDD and with 30 mg/kg 2,4,5-T + 2 μg/kg TCDD are highly significant ($P < 0.0027$).

dose of TCDD. This combination would correspond to a "contamination" of 3.3 ppm. When the dose of 2,4,5-T—and thereby the effect produced by this compound alone—is increased, even smaller doses of TCDD (0.1 μg/kg) lead to a detectable increase in the frequency of malformations. This combination would correspond to about 1 ppm. No significant potentiation can be observed when the dose of TCDD is lowered by another factor of 5 (to 0.02 μg/kg), corresponding to 0.2 ppm.

Although in such an experimental setup an effect by as little as 50 ng/kg TCDD can be detected, these data clearly show that a potentiating effect in this system cannot be obtained—even if highly teratogenic doses of 2,4,5-T are used—with a combination containing less than 0.5 ppm TCDD.

Further experiments were performed to get some information on what doses of TCDD are required to induce a detectable effect when just "nonteratogenic" doses of 2,4,5-T are used.

Under the conditions specified, 30 mg/kg was about a threshold dose. When this dose of 2,4,5-T is combined with 2 μg/kg TCDD (also a threshold dose), 66 ppm, a clear-cut potentiation can be observed (Fig. 7). The same dose of TCDD (2 μg/kg) increases the cleft palate frequency over the background also when half the 2,4,5-T dose is used (15 mg/kg), corresponding to 133 ppm. But 1/10 of the TCDD dose (0.2 μg/kg) does not induce any detectable effect with either of the doses 2,4,5-T used in this experimental series. This suggests that when just nonteratogenic doses of 2,4,5-T are used, there is no effedt with the system used if less than 10–20 ppm TCDD is present.

The results obtained with our experimental setup may give too high values since we have used neither the most sensitive strain of mice nor the malformation seen with the lowest dose in this strain (kidney abnormalities). It is furthermore interesting that with none of the combinations mentioned an increased fetomortality could be observed.

Cleft palate frequency induced by giving a combination of TCDD and other teratogens simultaneously on day 13 of gestation.—In these studies all the teratogens were given only once. Day 13 was chosen as the most convenient time of gestation (Fig. 4). In these studies, therefore, two of the teratogens were not given at the most sensitive phase of development.

Since 2,4,5-T cannot be considered a very powerful teratogen we have studied the possibility of an occurrence of a potentiation when TCDD is combined with other cleft palate-inducing teratogens. Special emphasis was placed on the differences in dose response characteristics for these agents. Results of such experimental series are compiled in Figure 8. In this study the cleft palate frequency was measured when just nonteratogenic doses (threshold doses) of the teratogens were combined with a threshold dose of TCDD (12 μg/kg).

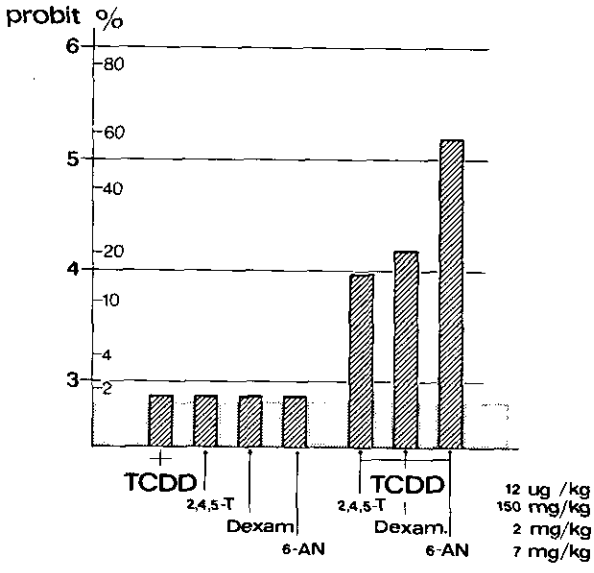

FIGURE 8. Cleft palate frequency induced by drug combinations. All drugs were given once on day 13 of pregnancy (TCDD and 2,4,5-T in rape-seed oil by stomach tube, all other drugs subcutaneously) at the doses indicated, which represent the just nonteratogenic (or threshold) doses as explained in Table 6. At least 12 litters were evaluated in each group. The data are given as percentage of the total fetuses evaluated per group (probit scale).

It can be seen that a potentiation occurs after a treatment with all the teratogens studied. The degree of potentiation varied somewhat; a combination of TCDD with 2,4,5-T resulted in about 15% cleft palate and about the same frequency was observed with a combination of TCDD with dexamethasone. With a combination of TCDD and 6-AN almost 60% of the mouse fetuses showed this teratogenic effect. It is interesting that the dose-response curve obtained with 6-AN is very steep when compared with those for 2,4,5-T or dexamethasone.

These data as well as data obtained with other combination (*21*) suggest that an especially pronounced potentiation is to be expected when two drugs showing both a rather steep dose–response curve are combined, while less potentiation is to be expected from the combined action of two drugs revealing a flat dose–response relationship. Of course, this prediction only holds as long as each drug does not act by interfering with the metabolism of the other. Accordingly, the TCDD–2,4,5-T pair should be expected to give a moderate degree of potentiation in the assay system used here.

Cleft palate frequency after treatment with a single dose of more than two teratogens.—We have, furthermore, performed studies to elucidate the possibility of TCDD potentiating the teratogenic action of other drugs when several such teratogens are given simultaneously in "subthreshold" doses. For these experiments all the drugs were given in doses of about half of the just nonteratogenic dose.

Figure 9 shows that neither a combination of 6-AN and dexamethasone nor the combination of the three drugs 6-AN, dexamethasone, and 2,4,5-T at this dose leads to an effect significantly different from that observed in controls. When TCDD is added to this drug combination as a fourth drug, however, at about half of the just nonteraogenic dose (6 μg/kg), a pronounced effect can be demonstrated. The cleft palate frequency is about 30%.

From these combination experiments and with regard to the model studied we wish to draw the following conclusions.

(1) When doses of TCDD much lower than the threshold dose are combined with teratogenic doses of drugs that are able to produce the same teratogenic effect, a potentiation may be expected, even with doses of TCDD as low as 1/20 to 1/50 of the threshold

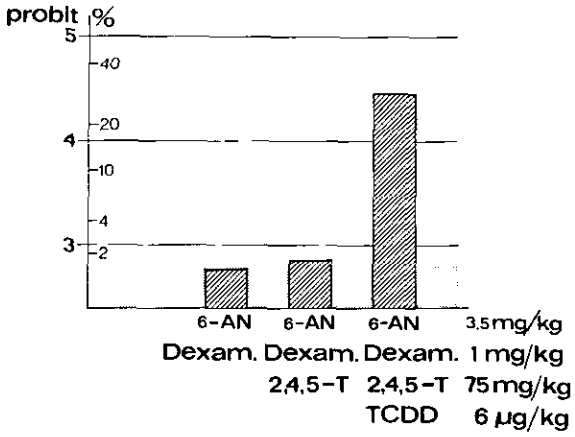

FIGURE 9. Cleft palate frequency induced by drug combinations. Experimental conditions as in Figure 8, except that more than two drugs were given simultaneously in some of the experiments. The doses given represent about half the threshold dose, as defined in Table 6.

dose (2 μg/kg).

(2) When just nonteratogenic doses of TCDD are combined with just nonteratogenic (threshold) doses of drugs which are able to produce the same teratogenic effect, a potentiation may be expected, the degree of which depends on tan α of the dose–response curve of the teratogens used.

(3) When two teratogenic drugs are combined at half the level of the threshold doses or lower, a significant effect is not to be expected unless both the teratogens exhibit very steep dose–response curves. In the case of TCDD a just significant effect is obtained with 6-AN but not in combination with 2,4,5-T, dexamethasone, or Endoxan.

(4) When TCDD at half the level of the threshold dose is combined simultaneously with three other teratogens, all at this dose, a clearcut potentiation is observed.

(5) Our results do not give any indication that in the system used TCDD in a dose of about 1/10 of the threshold dose would be able to induce a teratogenic effect, even in combination with up to three other teratogens simultaneously present in doses of half the threshold dose or lower.

All the foregoing conclusions are only valid as long as the drug metabolism of any of the teratogens used is not drastically altered during the combination studies.

Materials and Methods

For all experiments performed in this laboratory mice of the strain NMRI (purchased from Schwenke & Co., Bad Nauheim, Germany) weighing 29 ± 3 g were used. The animals were mated (20 females with 10 males per cage) for 2 hr and subsequently checked for vaginal plugs. The 24-hr period following the mating (8 A.M.) was called day 0 of pregnancy. Altromin R and tap water were given *ad libitum*.

The animals were sacrificed on day 18 of pregnancy and the number of viable fetuses was counted. Resorbed fetuses were regarded as dead. Litters with less than five implantation sites were not included in the evaluation. The average litter size was 11.1 ± 1.3 with this strain.

The results presented in this paper are based on the evaluation of about 1000 treated pregnant mice (ca. 11000 fetuses) and about 250 controls (ca. 2800 fetuses).

Embryotoxic effects have been evaluated in this paper as frequency per single litter or alternatively as percentage of the total of fetuses examined. Although the litter should be considered the experimental unit for statistical evaluations in teratological studies we feel that a calculation of the embryotoxic effects based on the total number of fetuses examined is justified, since a closer analysis of our data always has revealed that the occurrence of embryotoxic effects is distributed randomly (*14*). In all general calculations of our data litters showing more than 75% resorptions have not been included but were listed separately.

Embryolethal and teratogenic effects were statistically evaluated from the total percentages or from the frequencies occurring in each litter (M ± S.D.) by using a t-test (*22*) or a χ^2-test.

Pure TCDD (Lot No. 851:142-26) was kindly provided by the Dow Chemical Co., Midland, Michigan, U.S. This extremely toxic substance was handled with utmost care. The purity (gas chromatographic analysis) in-

dicated was 98.6%. The drug was dissolved in acetone or chloroform and vigorously mixed with rape-seed oil. Doses of 0.1 ml/10 g mouse were given by stomach tube.

Pure 2,4,5-T (Lot SHG) was kindly provided by C.H. Boehringer, Ingelheim, Germany. The dioxin content indicated was < 0.02 ppm. Since this preparation was synthesized by a special procedure it may be considered dioxin-free. The drug was dissolved in rape-seed oil by gently warming to 40° C and given to the mice by stomach tube (0.1 ml/10 g mouse). When 2,4,5-T and TCDD were given together, stock solutions were mixed before giving the solution (oil) to the experimental animals at a dose of 0.1 ml/10 g.

6-AN was purchased from Calbiochem (U.S.)

Dexamethasone (Fortecortin) was a gift of E. Merck, Darmstadt, Germany. It was given to the mice in aqueous solution by subcutaneous injection.

Pure cyclophosphamide (Endoxan) was a gift of Asta-Werke, Brackwede, Germany. The drug was given to the mice in aqueous solution by subcutaneous injection.

REFERENCES

1. Courtney, K. D. et al. Teratogenic evaluation of 2,4,5-T. Science **168**: 864 (1970).
2. Hofmann, H. T. Neuere Erfahrungen mit hochtoxischen Chlorkohlenwasserstoffen. Arch. Exp. Path. Pharmak. **232**: 228 (1957).
3. Kimmig, J., and Schulz, K. H. Chlorierte aromatische zyklische Äther als Ursache der sogenannten Chlorakne. Naturwiss. **44**: 337 (1957).
4. Higginbotham, G. R., et al. Chemical and toxicological evaluations of isolated and synthetic chloro derivatives of dibenzo-*p*-dioxin. Nature **220**: 702 (1968).
5. Teleky. Die Pernakrankheit (Chloraene). Klin. Wschr. **6**: 845 (1927); *ibid.*, **6**: 897 (1927); *ibid.*, **7**: 214 (1928).
6. Neubert, D., and Merker, H. J. Arch. Toxicol., in press.
7. Becker, D. The effect of folate overdose and of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on kidneys respectively livers of rat and mice embryos. Teratology, in press.
8. Buu-Hoi, N. P., et al. Organs as targets of "dioxin" (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) intoxication. Naturwiss. **59**: 174 (1972).
9. Fink, J., et al. in preparation.
10. Stolpmann, H.-J. in preparation.
11. Sparschu, G. L., Dunn, F. L., and Rowe, V. K. Teratogenic study of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in the rat. Toxical. Appl. Pharmacol. **17**: 317 (1970).
12. Sparschu, G. L., Dunn, F. L., and Rowe, V. K. Study of the teratogenicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in the rat. Food Cosmet. Toxicol. **9**: 405 (1971).
13. Courtney, K. D., and Moore, J. A. Teratology studies with 2,4,5-trichlorophenoxyacetic acid and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Toxicol. Appl. Pharmacol. **20**: 396 (1971).
14. Neubert, D., and Dillmann, I. Embryotoxic effects in mice treated with 2,4,5-trichlorophenoxyacetic acid and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Arch. Pharmacol. **272**, 243 (1972).
15. Schwetz, B. A., et al. Toxicology of chlorinated dibenzo-*p*-dioxins. Environ. Health Perspect. No. **5**: 87 (1973).
16. Wilson, J. G. et al. FDA Report: Report of the Advisory Committee on 2,4,5-T to the Administrator of the Environmental Protection Agency, May 1971.
17. Koehler, E., Barrach, H.-J., and Neubert, D. Inhibition of NADP dependent oxidoreductases by the 6-aminonicotinamide analogue of NADP. FEBS Letters **6**: No. 3,225 (1970).
18. Neubert, D., et al. In: Biochemical Aspects of Teratology. (Advances in the Biosciences, Vol. 6), Pergamon Press–Vieweg, Beaunschweig 1971, p. 575.
19. Barrach, H.-J. Inaugural Dissertation, Free University Berlin, 1973.
20. Barrach, H. J. Effect of 6-amino-nicotinamide on the glucose metabolism of embryonic tissue. In Metabolic Pathways in Mammalian Embryos during Organogenesis and Its Modification by Drugs, Free University Press, Berlin, 1970, p. 365.
21. Rothenwallner, A., Zens, P., and Neubert, D. Arch. Pharmacol., in press.
22. Patau, K.: Zur statistischen Beurteilung von Messreihen (eine neue *t*-Tafel). Biol. Zbl. **63**: 152 (1943).