

Results of *in Vivo* and *in Vitro* Studies for Assessing Prenatal Toxicity

by Diether Neubert,* Gudrun Blankenburg,* Ibrahim Chahoud,* Gabriele Franz,* Rainer Herken,* Michael Kastner,* Stephan Klug,* Joachim Kröger,* Ralf Krowke,* Constanze Lewandowski,* Hans-Joachim Merker,* Thomas Schulz,* and Ralf Stahlmann*

Examples of a combined approach using *in vivo* as well as *in vitro* methods for the assessment of prenatal toxicity are presented. The topics discussed include the analysis of the possible embryotoxic potential of valproic acid (VPA), female sex hormones, bis(tri-*n*-butyltin) oxide (TBTO), and acyclovir and the problem of supplementing *in vitro* systems with drug-metabolizing activity.

Introduction

Supplementation of the classical *in vivo* tests with recently developed *in vitro* techniques has greatly extended the scientific possibilities in prenatal toxicology (1-3). In this presentation we shall give some examples of such a combined approach as extensively used in our institute in recent years. We strongly feel that only the concerted use of *in vivo* methods, *in vitro* techniques, pharmacokinetic studies, and additional specific morphological (especially electron microscopic) and biochemical investigations will allow us to successfully tackle the important problems in revealing the principles and special aspects of prenatal toxicity in the years to come.

On the other hand, one should be aware of the possibility of producing "artifacts" *in vitro*; misinterpretation of the data may occur easily and must be avoided (4). One of the major difficulties still connected with *in vitro* methods is to distinguish general cytotoxic effects, which may be produced with any substance at a high enough concentration, from specific interferences with differentiation processes.

None of the culture methods available today are, alone, adequate for tackling the variety of problems and all of them have considerable limitations as well as advantages; the most suitable method has to be selected for solving a special problem.

Here we present results of studies supplemented with different culture techniques on some aspects of abnormal

prenatal development induced by valproic acid studied with the "whole-embryo" culture technique which we have modified for use on a larger scale; the question of a possible induction of abnormal heart development by some female sex hormones ("whole-embryo" culture technique); the problem of assessing a possible embryotoxic potential of bis(tri-*n*-butyltin) oxide (TBTO) from *in vivo* and *in vitro* data (limb bud assay); the problem of supplementing *in vitro* experimental test procedures with systems allowing (at least some type of) metabolic activation (organ cultures); assessing the possible embryotoxic potential of the virostatic agent: acyclovir, using a combined *in vivo/in vitro* approach ("whole-embryo" culture technique).

Modification and Standardization of the Whole-Embryo Culture Technique

Over the last 5 years we have systematically modified and standardized the technique of cultivating early post-implantation rat or mouse embryos as described by New and co-workers (5) so that it is now easier to use on a larger scale for toxicological studies. The main advantage of our method is the use of bovine serum as a culture medium instead of rat serum (6). This modification allows the study of a large number of samples under different experimental conditions using the same batch of serum. The conditions for whole-embryo culture have been modified in such a way (adjustment of gassing conditions, etc.) that the differentiation

*Institute of Toxicology and Embryopharmacology, Free University Berlin, Garystr. 5, D-1000 Berlin 33, TRG.

Table 1. Comparison of rat and bovine serum as culture medium and different gassing procedures.

Medium	YS, mm ^a	CR, mm ^a	Som	Prot, µg/ embryo	Score	ABN, %
Gassing I						
0 hr		5% O ₂	5% CO ₂			
20 hr		20% O ₂	5% CO ₂			
32 hr		40% O ₂	5% CO ₂			
100% Rat serum, n = 15		3.78	27.0	313.5	40.0	0
4.26 ^b		3.48	26.0	223.0	40.0	
4.02 ^b		3.12	26.0	206.5	39.0	
3.84 ^b						
86% Bovine serum + 14% Ty- rode,	4.08	3.34	25.0	241.5	37.0	25
n = 20	3.96	3.03*	24.0*	180.0*	35.5*	
	3.75	2.79	23.0	130.5	27.0	
Gassing II						
0 hr		10% O ₂	5% CO ₂			
36 hr		50% O ₂	5% CO ₂			
100% Rat serum, n = 20	4.68	3.48	28.0	304.0	40.0	5
	4.20	3.36	27.0	230.0	39.0	
	3.90	3.18	27.0	197.5	38.0	
86% Bovine serum + 14% Ty- rode,	4.98	3.54	27.0	250.0	39.0	0
n = 41	4.50*	3.36	26.0	230.0	38.0*	
	4.38	3.15	25.0	176.0	37.0	

^aYS = yolk sac diameter; CR = crown-rump length; Som = number of somite pairs; Prot = protein content.

^bMiddle rows are median values; the numbers in the bottom row represent the first quartile, the numbers in the top row represent the third quartile.

**p* = 0.01–0.05 (Mann-Whitney test).

achieved with bovine serum as culture medium is comparable to that obtained with rat serum (Table 1).

We have also developed and extensively used a simple scoring system for documentation of the data in a computerized form and for semi-quantifying normal or abnormal development in culture (6).

The outcome of the development *in vitro* and the conditions for analyzing the effect of chemicals in this system may be further improved and standardized by incubating the embryos at a temperature of 38.8°C instead of 37°C (Table 2); by adding 1 mL of buffer to 6 mL of serum (thus facilitating the addition of chemicals to the medium); and supplementing the bovine serum with methionine and purified hemoglobin (final concentrations: 7.5 mg/100 mL and 2.5 mg/mL, respectively). The addition of hemoglobin to the medium greatly improves the formation of hemoglobin within the developing embryos in culture.

Although a comparison of the development achieved in culture with that occurring in the intact organism is difficult because of the considerable variability *in vivo*, it seems fair to state that the technique now used—embryonic development achieved in culture—essentially resembles that seen *in vivo* with respect to crown-rump length, number of somite pairs, and score; the median protein content is about 85% of that of corresponding embryos developed in the living organism.

Abnormal Development Induced by Valproic Acid in Whole-Embryo Culture

From a large series of experiments performed with valproic acid (VPA) we shall present here only a few data to illustrate the versatile applicability of the

Table 2. Comparison of development of rats in whole-embryo culture at 37.0 ± 0.2°C and 38.8 ± 0.2°C incubation temperature (bovine serum).

Incubation temperature	YS, mm ^a	CR, mm ^a	Som ^a	Prot, µg/embryo	Score	ABN, %
37.0 ± 0.2°C n = 20	4.20 ^b	3.21	24.0	172.3	35.0	10
	3.96 ^b	2.94†	24.0*	141.0	33.0†	
	3.78 ^b	2.65	23.0	111.5	28.0	
38.8 ± 0.2°C n = 12	4.26	3.44	26.0	218.8	37.0	0
	4.08	3.30	25.0	185.5	35.0	
	3.90	2.91	24.0	151.0	34.0	

^aYS = yolk sac diameter; CR = crown-rump length; Som = number of somite pairs; Prot = protein content.

^bMiddle row are median values; the numbers in the bottom row represent the first quartile, the numbers in the top row represent the third quartile.

**p* = 0.01–0.5 (Mann-Whitney test).

†*p* ≤ 0.01 (Mann-Whitney test).

method. Abnormal development in whole-embryo culture induced by valproic acid was first described by Kao et al. (7) and has been extensively studied by Brown (8). Since the teratogenic action of VPA has predominantly been demonstrated *in vivo* with mice, it is interesting to note that typical defects seen in mice (predominantly defects in the head region) can also be observed *in vitro* with rat embryos—a species which has not been found so far to respond readily to VPA with the formation of CNS abnormalities *in vivo*. The susceptibility of the rat embryo to VPA *in vitro* can be shown on the macroscopic (Fig. 1) as well as the microscopic level (Fig. 2), especially at the head region and the somites.

We have shown (9) that one of the VPA metabolites (4-en-VPA) is similarly active in inducing abnormal development *in vitro* as VPA itself (Fig. 3 and Table 3), whereas another chemically similar derivative (2-en-VPA) is completely inactive in this respect. 4-en-VPA induces an additional type of gross structural abnormality *in vitro*, i.e., cardiac defects (Table 4). This example clearly shows that *in vitro* techniques are especially suited to assess toxic effects of metabolites in the absence of the original compound and under defined experimental conditions.

Since embryos are routinely standardized with re-

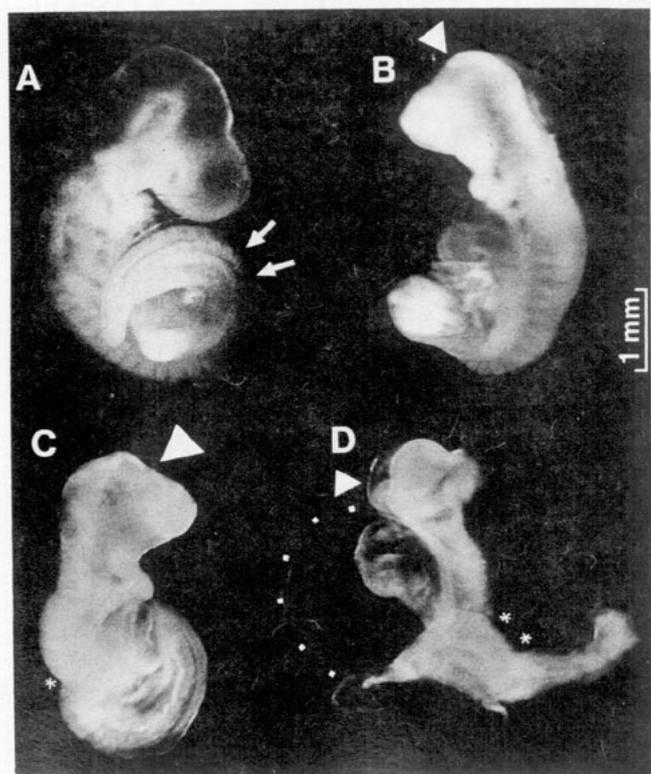


FIGURE 1. Effect of valproic acid (VPA) on rat embryos in whole-embryo culture (48-hr incubation): (A) control medium (6 mL bovine serum + 1 mL Tyrode's buffer); (B) + 0.6 mM VPA; (C) + 1.2 mM VPA; (D) + 1.8 mM VPA. Δ = head; ↑ = somites; □ = pericardial dilatation; * = incurvation of the spine.

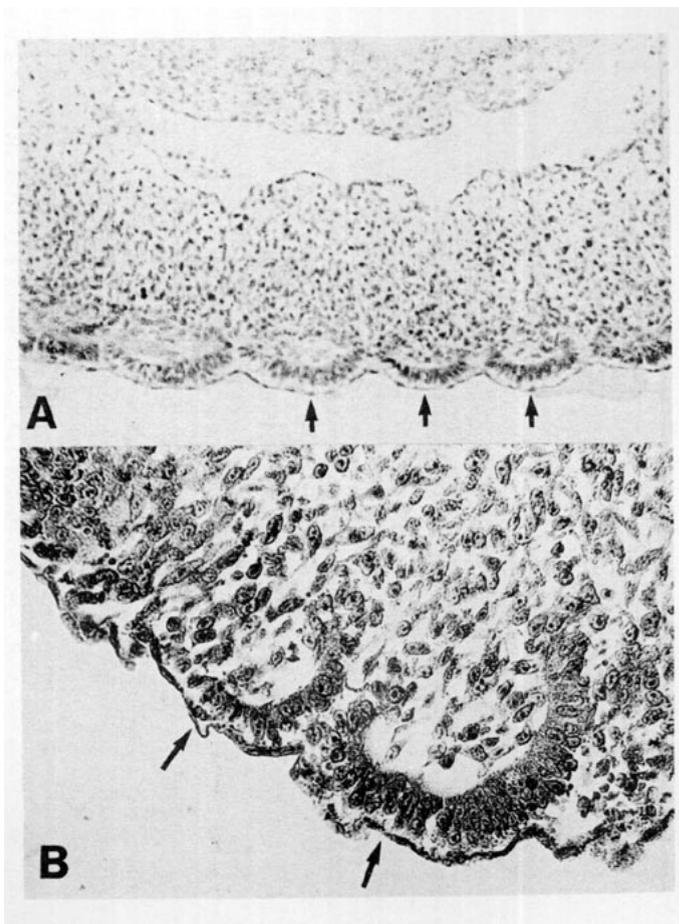


FIGURE 2. Effect of valproic acid (VPA) on rat embryos in whole-embryo culture (48 hr incubation): lateral sagittal section of an embryo; the alteration of the somite structures is obvious: (A) control medium; (B) + 0.6 mM VPA; ↑ = somites.

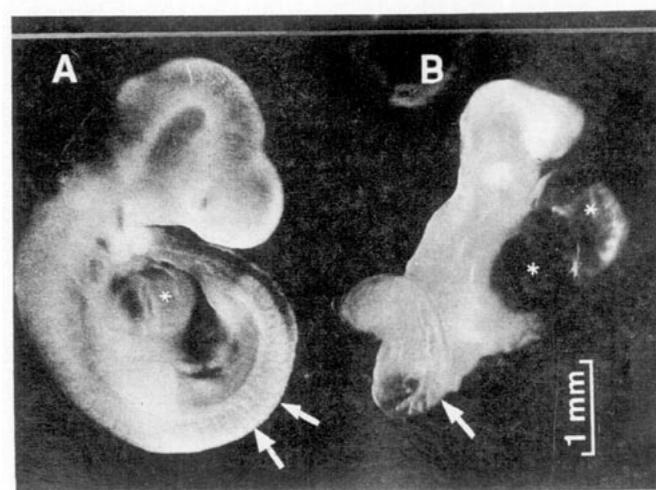


FIGURE 3. Effect of 4-en-VPA on the development of rat embryos in whole-embryo culture (48 hr incubation): (A) control medium; (B) + 1.8 mM 4-en-VPA; Δ = head; ↑ = somites; * = heart tube.

Table 3. Influence of 4-en-VPA (0.6 mM, 1.2 mM, and 1.8 mM) on the development of rat embryos in "whole-embryo" culture.

4-en-VPA, mm	YS, mm	CR, mm	Som	Prot, $\mu\text{g}/\text{emb.}$	Score	Abnormalities, %	Abnorm. per embryo
Control $n = 18$	4.77 ^b	3.61	26.2	249	37	0	0
	4.47 ^b	3.36	25.0	203	36		
	4.29	3.11	24.7	146	35.7		
0.6 mM $n = 18$	4.75	3.37	26.0	175	36.2	0	0
	4.50	3.15*	25.0	150 [†]	34.5		
	3.97 ^b	2.97	22.7	105	33		
1.2 mM $n = 15$	4.20	3.18	24.0	162	35	40	3 \times 1 Abn. 3 \times 2 Abn.
	4.02*	2.58 [†]	23.5 [†]	94 [†]	32 [†]		
	3.66	2.16	20.0	65	20		
1.8 mM $n = 14$	4.05	2.11	14.0	78	20	100	2 \times 1 Abn. 4 \times 2 Abn. 8 \times 3 Abn.
	3.90 [†]	1.89 [†]	14.0 [†]	69 [†]	16 [†]		
	3.84	1.50	12.0	51	15		

^aYS = yolk sac diameter; CR = crown-rump length; Som = number of somite pairs; Prot = protein content.

^bMiddle row are median values; the numbers in the bottom row represent the first quartile, the numbers in the top row represent the third quartile.

*Significant difference ($p = 0.01$).

[†]Significant difference ($p \leq 0.01$) to the control group.

Table 4. Type of abnormalities observed after treatment with VPA and 4-en-VPA in the whole embryo culture.

	Concentration, mM	n	Abnormality, %		
			Neural tube	Shape	Heart
Valproic acid	1.8	15	60	100	0
4-en-Valproic acid	1.8	14	85	92	64

spect to the developmental stage for *in vitro* studies when initiating the incubation, the variability of the development in culture is much smaller than the considerable variability of embryonic development within the living organism. This fact holds true for many culture methods.

Table 5 gives an example of the variability observed in mouse embryos *in vivo*. Such studies have been performed with various strains of rats and mice (10) and have given very similar results: there was always a considerable inter- and intralitter variation in the stages of normal prenatal development. This may be an explanation for the variability of outcome regularly seen when assessing embryotoxic effects *in vivo*. The example given in Table 5 concerns an inbred strain in which one would expect the highest degree of synchronization. The variability seen—as indicated by the somite stages—is considerable. For the 62 embryos evaluated, a median value of 40 somite pairs was found for 11-day-old embryos with a maximum of 44 and a minimum of 29 somites.

In contrast to this, the high degree of reproducibility of an *in vitro* system is shown, for example, for the morphogenetic differentiation of mouse limb buds in culture (Fig. 4). Because the explants can be standardized at the initiation of the culture (according to the somite stage of the embryos: ± 1 somite stage) all the limbs can be made to develop in a synchronized manner.

The *in vitro* techniques allow the exposure of the

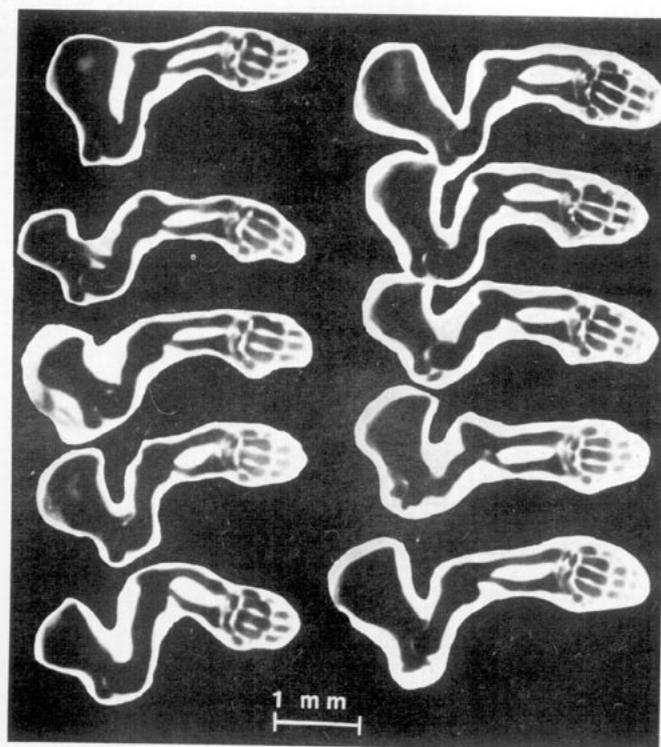


FIGURE 4. Example of the uniform development of explants in an organ culture system. Limb buds from 12-day-old mouse embryos cultured for 6 days (11).

explants to the toxic agent for a limited period only; before and after this period the cultivation is allowed to proceed in normal culture medium (12). We have used the whole-embryo culture technique to reveal the period of the highest susceptibility of the rat embryo to VPA. The results of our studies indicate that 9.5-day-old rat embryos are most susceptible to VPA 12 to 18 hr after the initiation of the culture (Table 6). This would cor-

Table 5. Example of the intra- and inter-litter variability of a developmental stage (pairs of somites) in C57Bl mice.^a

Litter no.	No. of somite pairs																Litter size
	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	
1						1				1			1				3
2						1				1		1					3
3											1			1	1	1	4
4										1	1	1		2			5
5	1				1				3	1							6
6												1	2	2	1		6
7										1		1	2	2		1	7
8												1		2		3	6
9													3	2	1		6
10						1					2	1	2		1		7
11									3	3	1	2					9
Percentage distribution	2				2	5			10	13	7	15	16	18	7	8%	(n = 62)

^aSomites were counted on day 11 of gestation (between 13⁰⁰ and 15⁰⁰).

Table 6. Effect of a short-term exposure (6 hr) to valproic acid (1.8 mM) in the culture medium at different developmental phases on growth and development of rat embryos *in vitro*.

Exposure	YS, mm ^a	CR, mm ²	Som ^a	Prot, µg/ embryo ^a	Score ^a	Abnormalities, %	Abnorm. per embryo
Control	4.68	3.42	27.3	220	37		
n = 35	4.44	3.24	26.0	175	36	0	0
	4.14	3.12	24.0	134	35		
	4.50	3.18	26.0	159	36		
0-6 hr n = 21	4.38	2.94	24.0	123	34	14	3 × 1 Abn.
	4.17	2.76	23.0	110	29		
	4.26	3.12	24.0	159	27		
12-18 hr n = 15	4.02†	2.82	23.0	140	25†	80	6 × 1 Abn. 5 × 2 Abn. 1 × 3 Abn.
	3.78	2.70	20.7	103	23		

^aYS = yolk sac diameter; CR = crown-rump length; Som = number of somite pairs; Prot = protein content.

^bMiddle row are median values; the numbers in the bottom row represent the first quartile, the numbers in the top row represent the third quartile.

† Significant difference ($p \leq 0.01$) between 0-6 hr and 12-18 hr.

respond to day 10.0 to 10.3 of gestation in the living organism.

Studies on the Possible Potential of Female Sex Hormones to Induce Abnormal Cardiac Development in Culture

There has been a considerable argument over the question whether estrogens or gestagens have a teratogenic potential in man outside the genital tract and, especially, whether they may induce cardiac abnormalities (13-17). The data of one of the main studies providing some evidence in this direction (17) have been doubted in their reliability (18) and apparently cannot be used to provide evidence in this direction.

Since the problem cannot be solved by epidemiological studies we attempted to obtain further clues on a possible teratogenic potential by testing the effects of such female sex hormones on the heart development of rat embryos in the whole-embryo culture. With this system the entire period of early heart development can be monitored. Furthermore, extremely high concentra-

tions of these substances may be evaluated *in vitro*, whereas *in vivo* the doses to be tested are very limited due to the early occurrence of embryomortality in rodents. The whole-embryo technique has been successfully used before to analyze the action of retinoic acid on heart development in culture (19).

With the presently available techniques, it is difficult to study development beyond day 12.5 of gestation in the rat. Therefore, possible effects on later stages of cardiac development (e.g., closure of the ventricular septum) cannot completely be ruled out.

When testing a large number of estrogens and gestagens using whole-embryo culture (Tables 7 and 8), we did not find any indication of the ability of these agents to interfere with cardiac development in rat embryos. The studies were performed with 9.5- as well as with 10.5-day-old rat embryos cultured for 48 hr. All the embryos have been evaluated histologically, in each case assessing serial sections. At extremely high concentrations (> 1 µg/mL) most of these substances produced necrosis in the embryo, predominantly at the CNS sites. Even in these cases cardiac development was unimpaired (Fig. 5). Taken together with all the epidemiological evidence available to date (20,21), our data do

Table 7. Evaluation of the effect of female sex hormones on cardiac development in whole-embryo culture (48-hr incubation; culture initiation: day 9.5).

	YS, mm ^a	CR, mm ^a	Som ^a	Normal	Retarded	Abnormal
Controls <i>n</i> = 27	5.0	3.5	28.0	25	1	1 ^b
	4.5	3.3	27.0			
	4.2	3.0	26.0			
β-Estradiol (3 μg/mL) <i>n</i> = 9	4.7	3.3	26.5	8	1	—
	4.6	3.2	26.0			
	4.4	3.0	24.0			
17-α-Ethinylestradiol (10 μg/mL) <i>n</i> = 15	7.8	3.6	26.0	10	3	2 ^b
	4.5	3.2	25.0			
	4.4	3.0	24.0			
DES (10 μg/mL) <i>n</i> = 14	4.8	3.1	26.5	8	4	2 ^b
	4.1	2.8	26.0			
	3.9	2.4	25.2			
Progesterone (1 μg/mL) <i>n</i> = 11	4.5	3.5	28.0	10	1	—
	4.4	3.3	27.0			
	4.2	3.2	26.0			
19-Norethisteron (3 μg/mL) <i>n</i> = 8	5.1	3.9	30.0	8	—	—
	4.5	3.2	27.0			
	4.3	3.0	27.0			

^aYS = yolk sac diameter; CR = crown-rump length; Som = number of somites.

^bNo cardiac abnormalities.

Table 8. Evaluation of the effect of female sex hormones on cardiac development in whole-embryo culture (48-hr incubation; culture initiation: day 10.5).

	YS, mm ^a	CR, mm ^a	Som ^a	Normal	Retarded	Abnormal
Controls <i>n</i> = 12	5.5	5.0	29.0	12	—	—
	5.5	4.7	28.0			
	5.2	4.2	27.3			
β-Estradiol (3 μg/mL) <i>n</i> = 12	5.5	4.7	30.0	9	—	—
	5.4	4.5	29.0			
	5.0	4.2	28.5			
17-α-ethinylestradiol (10 μg/mL) <i>n</i> = 6	5.6	4.6	29.3	6	—	—
	5.3	4.2	27.5			
	4.4	3.9	24.0			
DES (3 μg/mL) <i>n</i> = 6	5.5	4.4	28.0	6	—	—
	5.2	4.3	27.5			
	5.0	4.1	26.8			
Progesterone (3 μg/mL) <i>n</i> = 9	5.5	5.0	28.5	8	1	—
	5.2	4.4	27.0			
	5.2	4.1	26.0			
19-Norethisteron (3 μg/mL) <i>n</i> = 8	5.5	4.6	28.3	8	—	—
	5.5	4.5	27.5			
	5.4	4.2	26.8			

^aYS = yolk sac diameter; CR = crown-rump length; Som = number of somites.

^bNo cardiac abnormalities.

not support the speculation that female sex hormones interfere with cardiac development.

Studies on Assessing the Teratogenic Potential of TBTO*

Bis(tri-*n*-butyltin) oxide (TBTO) is a molluscicide and fungicide which is widely used. Its application may in-

crease in the future. It is, therefore, of importance to learn whether this substance possesses a potential to interfere with prenatal development. Again, it was attempted to solve this problem using a combined *in vivo/in vitro* approach. In collaboration with WHO we have performed *in vivo* experiments in mice. From these studies (22) it can be concluded that TBTO seems to exhibit embryotoxic and fetotoxic effects only at doses

*The term "teratogenic" is defined differently by different investigators in the field. Therefore, it is so ill defined that its significance for a toxicological risk assessment is small. It may even be misleading (e.g., for lawsuits). If possible, it should be avoided and replaced by

stating which kind of alteration is observed (specific congenital gross structural abnormality, special dysfunction, etc.). If we use the term at all it is for us synonymous with "inducing congenital gross structural abnormalities," not including certain "minor" abnormalities, anomalies, signs of retardation, or dysjunctions.

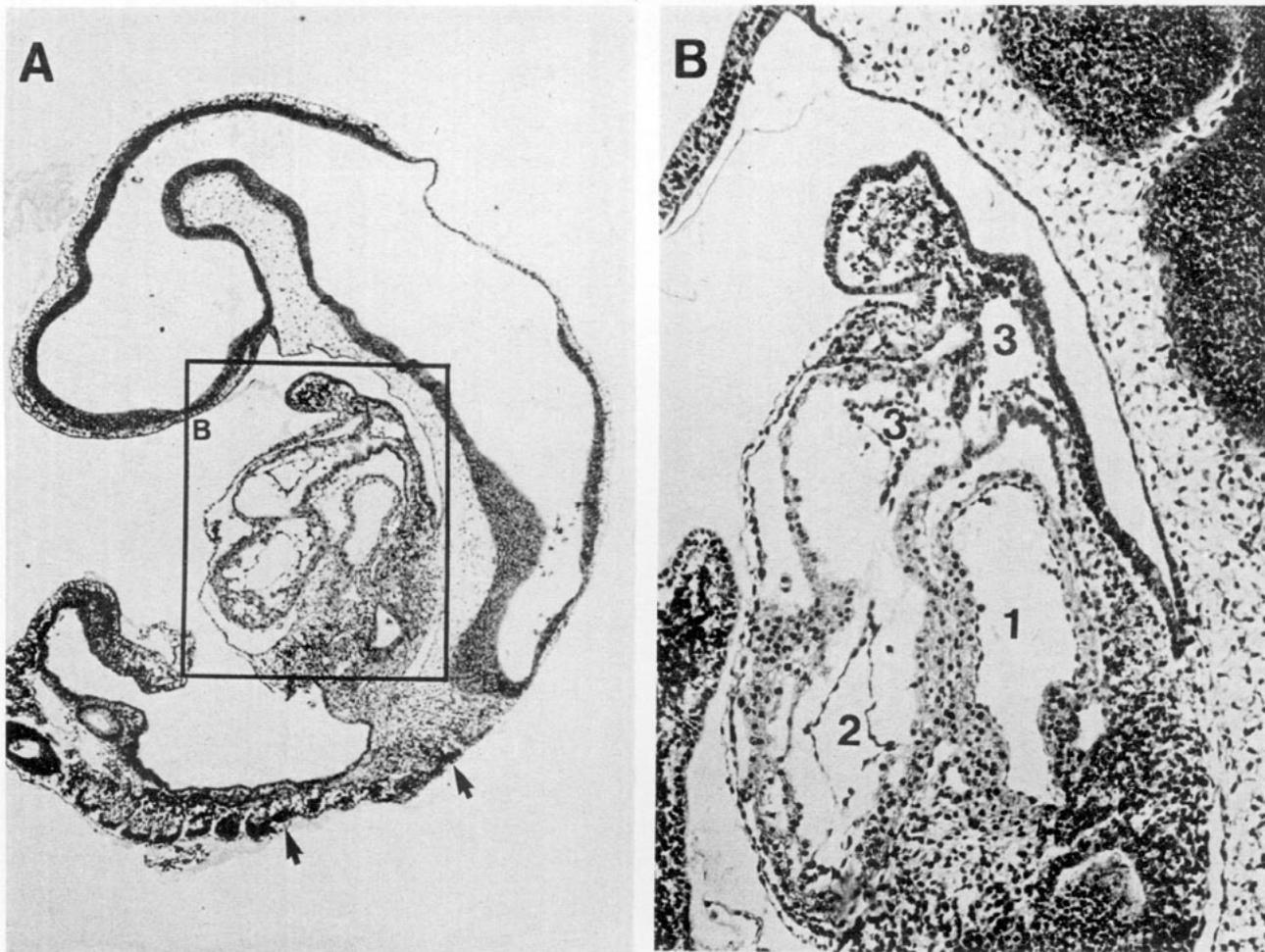


FIGURE 5. Rat embryo after 48-hr cultivation; treatment with 10 µg/mL 17-α-ethinylestradiol; culture started on day 9.5 of gestation: (A) sagittal section (↑) necroses in somites; (B) magnification of the heart: unimpaired development showing (1) atrium commune; (2) ventriculus communis; (3) tuncus arteriosus.

which are close to those producing maternal toxicity (Table 9).

The embryotoxicity and fetotoxicity of TBTO is compared with maternal toxicity (mortality) in Table 10. From these data it is obvious that embryotoxicity with this substance occurs close to maternal toxicity. The procedure of calculating ratios of relative embryotoxicity—as we have suggested (23)—requires the estab-

Table 10. Relative embryotoxic risk factors for TBTO.

	Risk factor	
Fetal LD ₅₀ /maternal LD ₅₀	≅ 30/74	≅ 0.41
Fetal LD ₂₀ /maternal LD ₂₀	> 20/45	> 0.44
ED ₅₀ cleft palate/maternal LD ₂₀	30/45	= 0.67
ED ₁₀ cleft palate/maternal LD ₂₀	> 10/45	> 0.22

Table 9. Embryo/fetotoxicity of TBTO in mice.

	TBTO dose			
	Controls	5 µL/kg	10 µL/kg	30 µL/kg
Live fetuses	1154	248	204	21
% Resorptions	9.1	9.8	9.7	58.8
Average weight of fetuses, g	1.13 ± 0.12	1.19 ± 0.08	1.10 ± 0.15	0.91 ± 0.19
Cleft palates, %	8 (0.7%)	4 (2%)	14 (7%)	10 (48%)
Minor skeletal deformities	85 (7%)	28 (11%)	22 (10%)	8 (38%)

lishment of dose-response relationships. Furthermore, maternal toxicity must be evaluated in pregnant animals of the same strain. It is completely worthless with such an approach to rely on data obtained from nonpregnant animals or from the literature as has been suggested by some investigators, since the variability of estimating the LD₅₀ value with different strains and in different laboratories is well known, and data may vary by one order of magnitude.

Subsequent studies using the limb bud organ culture system revealed an exceptional capability of this substance to interfere with morphogenetic differentiation *in vitro* (Table 11): concentrations as low as 50 nM (30

Table 11. Effect of TBTO on limb bud differentiation *in vitro*.

TBTO concentration, nM	Interference with development ^a	
	day 11	day 12
17	0	0
50	+	0
170	++	+
500	+++	++
1700	-	-

^a0 = no effect; + = clear-cut effect; ++ = strong effect; +++ = clearly abnormal differentiation; - = completely inhibited growth and differentiation.

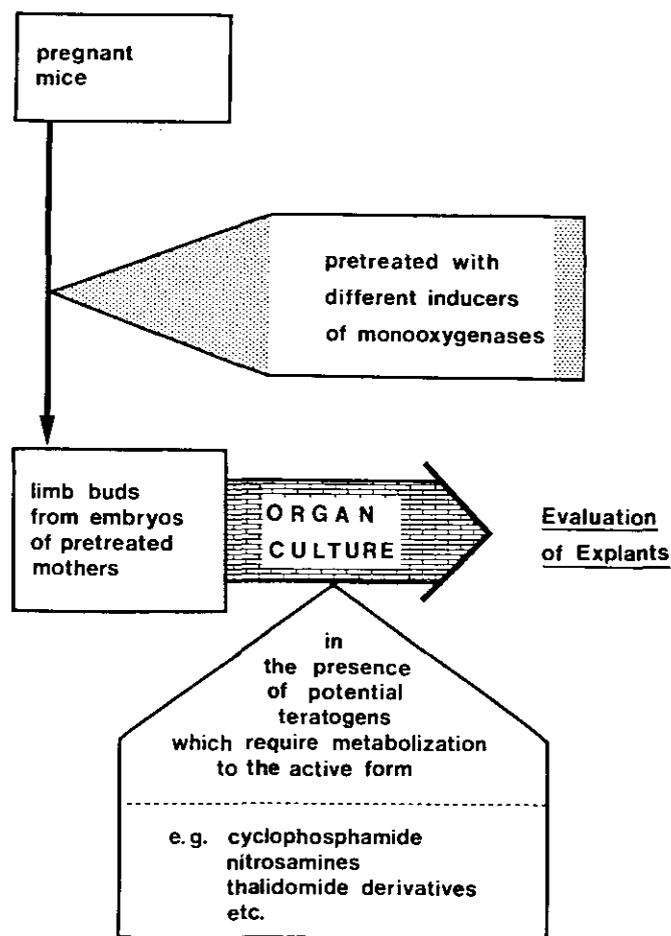


FIGURE 6. Experimental design for studying substances which require metabolic activation within the explants to interfere with morphogenetic differentiation.

ng/mL) strongly inhibited the development in culture (24). This is one of the lowest concentrations of a substance ever found to interfere with development in this organ culture system.

When measuring the tin content within the embryonic tissues of mice injected with TBTO, we found concentrations (22) which, if caused by original TBTO, should be more than sufficient to induce embryotoxicity. We are presently involved in an attempt to analyze the cause of this apparent discrepancy. One explanation

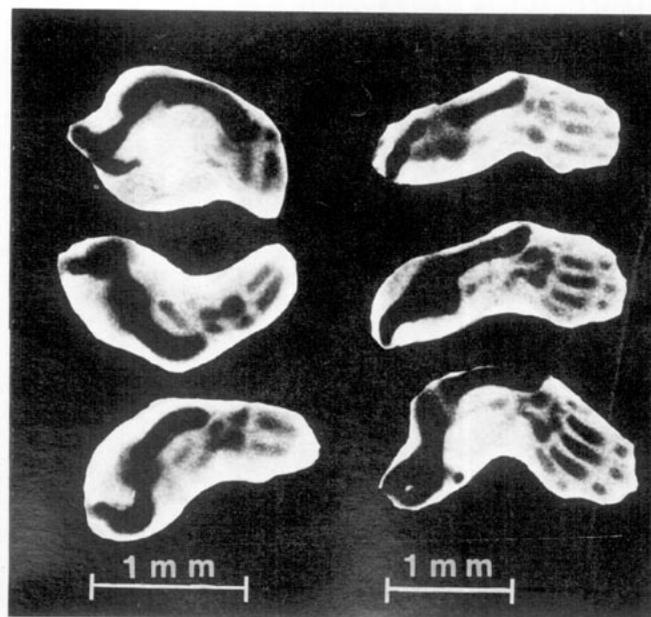


FIGURE 7. Effect of cyclophosphamide on limb bud differentiation in culture subsequent to induction of monooxygenase activity with PCBs (3×200 mg/kg Aroclor A60) *in utero*. Hindlimbs of 12-day-old mouse embryos: (a) controls; (b) + cyclophosphamide *in vitro*.

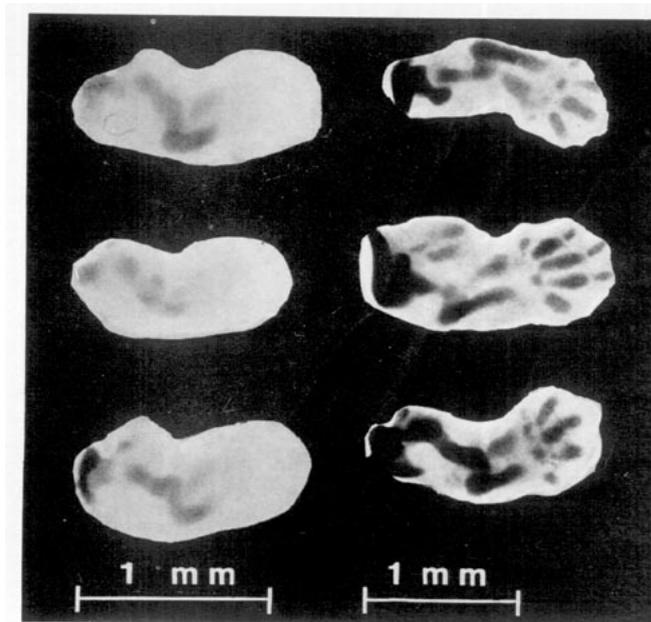


FIGURE 8. Effect of dimethylnitrosamine (DMNA) on limb bud differentiation in culture subsequent to induction of monooxygenase activity with β -naphthoflavone (3×150 mg/kg) *in utero*. Forelimbs of 11-day-old mouse embryos: (a) controls; (b) + DMNA *in vitro*.

could be that a metabolite of TBTO with a lower embryotoxic potential accumulates within the embryo. To demonstrate this, the experiments have to be supplemented with further pharmacokinetic studies and an assessment of the (presumably lower) potential of such

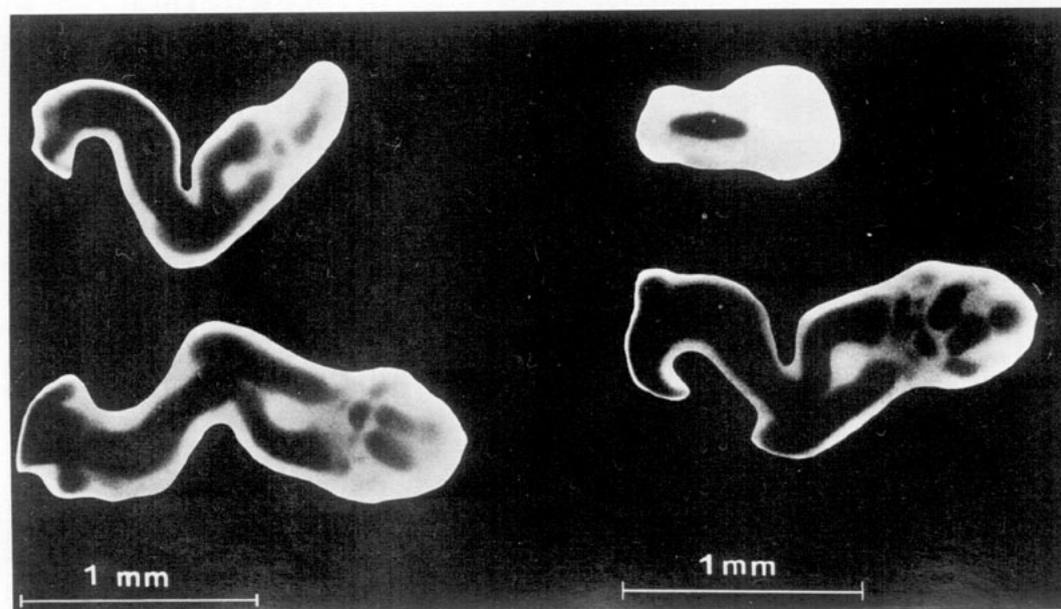


FIGURE 9. Mouse limb buds on day 11 of gestation after 4 days of culture. (Left) limb buds in assays containing a reconstituted cytochrome P-450 system, NADPH, and 100 µg/mL cyclophosphamide; (right) control assays containing the same components minus NADPH or cyclophosphamide; (top row) cytochrome P-450 concentration: 90 pmole/mL; (bottom row) cytochrome P-450 concentration: 9 pmole/mL.

Table 12. Species differences in the development of monooxygenases.^a

Developmental stage	Rat ^b			Marmoset ^b		
	Cou. 450	Res. 40	Mo. 3000	Cou. 263	Res. 179	Mo. 2000
- 2 days	2	—	—	—	—	80-90
1 day	8	—	—	—	—	—
Birth +	—	20	—	46	—	—
1 day	—	—	—	—	—	—
2 days	—	78	—	100	—	—
3 days	20	—	—	—	—	—
1 week	64	100	—	—	—	95
2 weeks	100	—	—	—	—	—
1 month	—	—	—	—	6	100
2 months	—	—	—	—	100	—

^aCou. = ethoxycoumarin *O*-deethylase; Res. = ethoxyresorufin *O*-deethylase; Mo. = ethylmorphine *N*-demethylase.

^bThe data are given in (%) of adult enzyme activity. 100% = pmole/mg protein-min.

^cAlready 20% on day 60 of gestation.

— = not detectable.

a TBTO metabolite to interfere with morphogenetic differentiation *in vitro*.

Problems Involved in Supplementing *In Vitro* Systems with Xenobiotic-Activating Capacities

One of the disadvantages of many *in vitro* systems utilizing embryonic rodent tissues is the inability of these explants to respond to metabolic activation. Especially with organ culture systems, the simple addition of microsomal fractions (or S-9-mix) is completely unsatisfactory, since these crude fractions strongly inter-

fere with development in culture. Another approach has been to perform organ cultures in the presence of "feeder cells" capable of at least some functions of drug metabolism. While this latter approach has been successfully used in a few laboratories (25), it also has a number of limitations, especially since the spectrum of the type of metabolic activation attainable with the supplementing cells (including hepatocytes) is at present quite limited.

For a number of years we have been trying two other approaches: to induce the capacity for metabolic activation within the explants to be used for the organ culture studies, and to isolate defined cytochrome P-450 fractions and to add a reconstituted system to the culture medium. The first approach—if feasible—would

Table 13. Effect of acyclovir and physiological deoxynucleosides (2'-deoxyguanosine, 2'-deoxyadenosine) on growth and development of 9.5 day-old rat embryos in culture ($\Sigma n = 213$).

	YS, mm ^a	CR, mm ^a	Som ^a	Prot, $\mu\text{g}/\text{embryo}^a$	Score	ABN, %
Control <i>n</i> = 44	4.56 4.32 (100%) 4.20	3.60 3.36 (100%) 3.18	27.0 26.0 (100%) 25.0	283.5 234.5 (100%) 176.0	38.0 37.0 35.25	0
25 μM Acyclovir <i>n</i> = 27	4.50 4.26 (99%) 4.08	3.30 3.06 (91%)* 2.88	26.0 25.0* 23.0	196.0 150.0 (64%)* 123.5	36.0 36.0* 24.0	0
50 μM Acyclovir <i>n</i> = 18	4.51 4.35 (101%) 4.17	3.36 3.15 (94%)* 2.88	27.0 26.0 24.0	175.0 130.0 (55%)* 110.0	38.0 34.5* 32.75	28%
100 μM Acyclovir <i>n</i> = 19	4.80 4.56 (106%)* 4.38	3.36 3.00 (89%)* 2.82	27.0 26.0 25.0	174.5 137.5 (59%)* 122.3	33.0 32.0* 31.0	95%
200 μM 2'-Desoxyguanosine <i>n</i> = 16	4.65 4.53 (105%) 4.33	3.76 3.45 (103%) 3.25	27.75 26.5 26.0	284.0 252.0 (108%) 225.0	38.0 37.0 36.0	0

^aYS = yolk sac diameter; CR = crown-rump length; Som = number of somite pairs; Prot = protein content; ABN = abnormalities.

^bMiddle rows are median values; the numbers in the bottom row represent the first quartile; the numbers in the top row represent the third quartile.

*Significantly different from control group at $p < 0.05$ (Mann-Whitney test).

Table 14. Influence on acyclovir on the ear development of 9.5-day-old rat embryos *in vitro*.

Ear development	Acyclovir concentration					
	Control, <i>n</i> = 44	10 μM , <i>n</i> = 19	25 μM , <i>n</i> = 27	50 μM , <i>n</i> = 18	100 μM , <i>n</i> = 19	200 μM , <i>n</i> = 21
Rec. dors. present, otic vesicle closed	71%	58%	26%	28%	10.5%	—
Otic vesicle closed	18%	42%	30%	5.5%	10.5%	—
Otic vesicle slightly open	11%	—	30%	33%	37%	9.5%
Otic vesicle half open	—	—	3%	5.5%	10.5%	19%
Otic pit	—	—	11%	28%	31.5%	71.5%

Table 15. Overview of the histological results in the different test groups.

Tissue	Control; 10 μM Acyclovir; 200 and 500 μM 2'-desoxyguanosine	25 μM Acyclovir	50 μM Acyclovir	100 μM Acyclovir	200 μM Acyclovir
Neuroepithelium	Multilayered	Multilayered	Multilayered; partly monolayered	Monolayered; Mitosis \downarrow Necroses \uparrow	Monolayered; Mitosis $\downarrow\downarrow$ Necroses $\uparrow\uparrow$
Central channel	0	0	Slightly dilated	Dilated; Necroses \uparrow	Dilated \uparrow , Necroses $\uparrow\uparrow$
Telencephalon	0	0	Some poorly developed or completely missing	Poorly developed \uparrow or completely missing	Poorly developed \uparrow or completely missing
Ear	Otic vesicle closed Cells cylindrical	Otic vesicle not completely closed Cells cylindrical	Otic pit partly Necroses; Cells cylindrical	Otic pit Necroses \uparrow Cells partly cubical	Ear plate, little cell material; Necroses $\uparrow\uparrow$; Cells cubical
Somites	Regular	Regular	Partly irregular; "Frayed"; Necroses	Irregular; "Frayed"; Necroses $\uparrow\uparrow$	Irregular \uparrow ; "Frayed" \uparrow ; Necroses $\uparrow\uparrow$

be superior to all the other approaches, since the potentially toxic substances would be metabolically activated by the target cells themselves. This is especially important if short-lived active metabolites are to be expected.

So far, only attempts that induce a special subtype of monooxygenase activity: AHH = aryl hydrocarbon hydroxylase, a cytochrome P-448-dependent enzyme system, have been convincing. We have been able to induce an AHH type of activity (e.g., benzo[a]pyrene

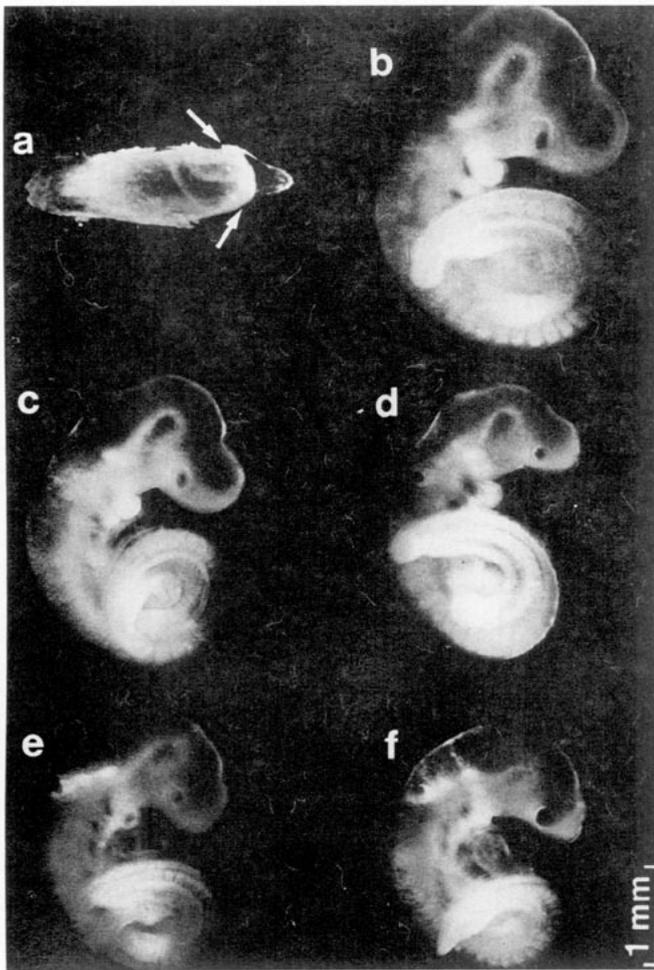


FIGURE 10. Effect of acyclovir in whole-embryo culture: (a) 9.5-day-old rat embryo (\downarrow) in its membranes at the beginning of the culture; (b)–(f) rat embryos after 48 hr of culture in bovine serum with test substances added to the medium. Culture in the presence of: (b) 200 μ M 2'-deoxyguanosine (development corresponding to controls); (c) 25 μ M, (d) 50 μ M, (e) 100 μ M, and (f) 200 μ M acyclovir, respectively, increasing extent of interference with prenatal development.

hydroxylase) at the stage of organogenesis in various rodent embryos (26). It has been found to be much more difficult to induce typical cytochrome P-450 activities in embryonic tissues of rodents. Although some such enzyme activities may be detectable with the highly sensitive methods available today (27), all the activities studied up till now remained so low—even after attempts to enhance the activities by various inducers—that a reproducible and exploitable monooxygenase activity sufficient for routine test purposes *in vivo* could not be obtained.

By using very high substrate concentrations *in vitro*, it is feasible to test the potential to induce abnormal development with explants or cells subsequent to an enzyme induction *in vivo*. With this experimental set-up apparently only some subtypes of cytochrome P-450-dependent activities can be induced within the embryonic tissues of rats or mice. We have been successful

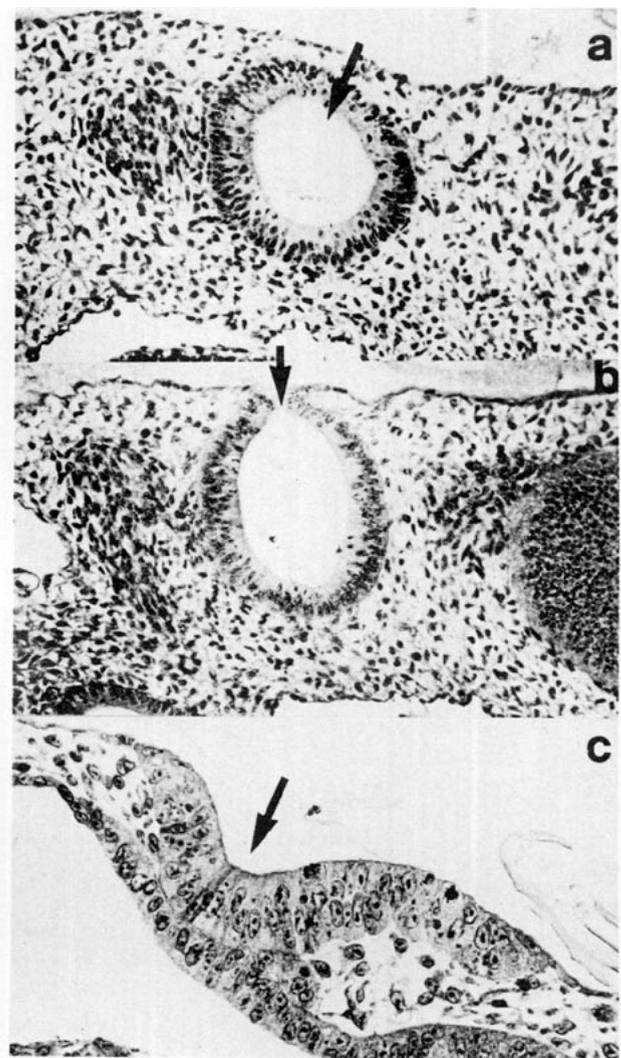


FIGURE 11. Effect of acyclovir on the development of the ear in rat embryos after 48-hr cultivation (whole-embryo culture) starting with day 9.5 of gestation: (a) untreated control: showing a closed otic vesicle (\downarrow); (b) 100 μ M acyclovir: otic vesicle (\downarrow) not yet completely closed; (c) 200 μ M acyclovir: otic placode with incipient invagination (\downarrow), greatly reduced cell number.

with this experimental design (see schematic presentation in Fig. 6) in studying abnormal development in organ cultures with cyclophosphamide and dimethylnitrosamine using explants of embryos after induction of monooxygenase activities with PCBs (Aroclor A60) or β -naphthoflavone (28,29) *in utero*. Typical examples of such an approach are shown in Figures 7 and 8.

With respect to the second approach, after many years of attempts and failure we have now succeeded in obtaining enzyme fractions of very high specific activities which are tolerated by the explants in culture (30). Using cyclophosphamide as a model substrate, a pronounced and clear-cut metabolic activation is achieved with the addition of 10 nM cytochrome P-450 fractions from various sources and the necessary com-

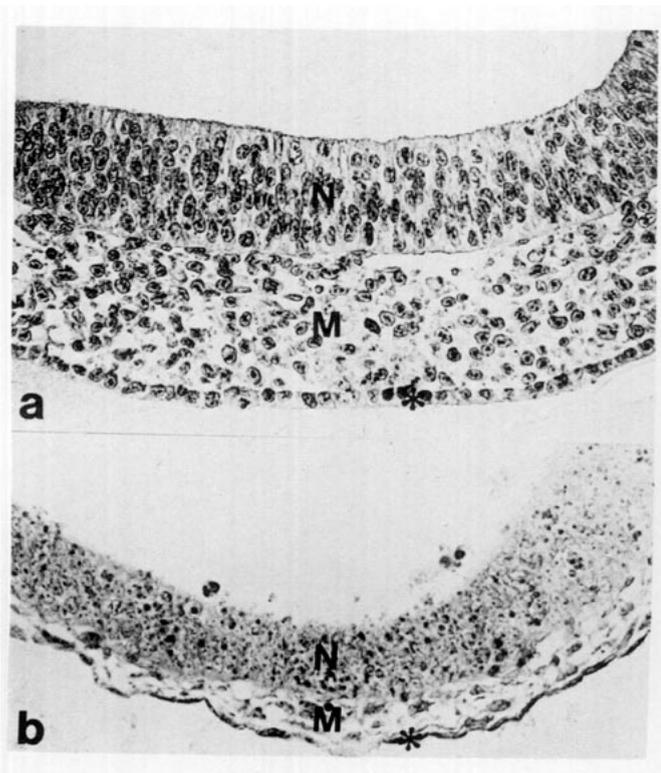


FIGURE 12. Effect of acyclovir on development of neuroepithelium in rat embryos after 48-hr cultivation (whole-embryo culture) starting with day 9.5 of gestation: (a) untreated control: neuroepithelium in the region of the telencephalic vesicle (N), mesenchyme (M), ectodermal epithelium (*); (b) 200 μ M acyclovir: numerous necroses in the neuroepithelium (N), mesenchyme (M) decreased in amount, thin ectodermal epithelium (*).

plementing components (Fig. 9). The system developed has itself a low toxicity since all the components have been extensively purified and it can now be used for many kinds of *in vitro* systems.

As a word of caution we would like to stress that by adding drug-metabolizing capacity to a culture medium it is possible to produce artifacts on the target cells (4), i.e., results with products of metabolic activation which have no relevance to the situation *in vivo*. Therefore, a single result obtained with such a system has little value with respect to toxicological risk assessment unless it is backed with additional results. This is a limitation with almost all *in vitro* systems. Many results reported in the literature obtained with substances such as thalidomide *in vitro* are not to be interpreted for this reason. In such situations, we have suggested (31) analyzing groups of chemically very similar derivatives from which it is known that they exert different biological effects *in vivo* in order to verify whether the result obtained is biologically meaningful.

It should be remembered that considerable species differences exist with respect to the prenatal and perinatal development of the activity of drug-metabolizing monooxygenases. These have to be considered if an ex-

trapolation of data from one species to another is attempted. While the activity of cytochrome P-450-dependent monooxygenases is extremely low or absent in rodent embryos and early fetuses, such activity may be detectable in the livers of primates at rather early fetal stages (32).

Table 12 gives some examples of differences in perinatal development of some monooxygenases in rats and marmosets. Because of practical reasons we find the marmoset to be a primate species which is especially suited for special studies in prenatal toxicity, and therefore some data are included here. While typical cytochrome P-450-dependent monooxygenases (e.g., ethylmorphine demethylation) are already detectable at day 60 of gestation (duration of pregnancy 140 days) in the marmoset (*Callithrix jacchus*), the activity of other enzymes (predominantly such of the cytochrome P-448 type) may develop even later than in the rat.

With the technique of adding reconstituted monooxygenases to *in vitro* systems, we intend to study systematically the effect of monooxygenases of different species—including those of primates—after induction by different agents in various culture systems.

Assessment of Possible Teratogenic Potential of Acyclovir Using an *In Vitro/In Vivo* Approach

One of the best examples for the use of a combined *in vitro/in vivo* approach comes from recent studies performed in our laboratory to elucidate a possible embryotoxic potential of the virostatic agent acyclovir which is used for the treatment of herpes infections. Segment-II tests performed before on a routine basis (treatment on days 6–15 of pregnancy) have not shown any indication for an embryotoxic potential of this agent (33). Since the doses that could be tested in the experimental animals *in vivo* are limited (because of an interfering nephrotoxicity in these species), we initiated *in vitro* studies using the whole-embryo culture technique.

The substance was clearly able to interfere with embryonic development *in vitro* (Table 13 and Fig. 10) at concentrations exceeding 25 μ M (34). Serum concentrations measured in man under extreme therapeutic conditions (IV application) approach 100 μ M; serum peak concentrations to be expected after oral medication with the dose regime used today are in the range of 2 μ M.

Abnormal development as shown *in vitro*—besides a general retardation, especially at higher doses—predominantly concerns the shape of the head; most susceptible to the action of acyclovir is the development of the ear (Tables 14 and 15 and Fig. 11). Histologically, necrosis can be seen in several parts of the embryos, e.g., neuroepithelium, somites, ear, etc. (Table 15 and Fig. 12).

The question arose why the substance had not been found to induce abnormal development *in vivo*. Two

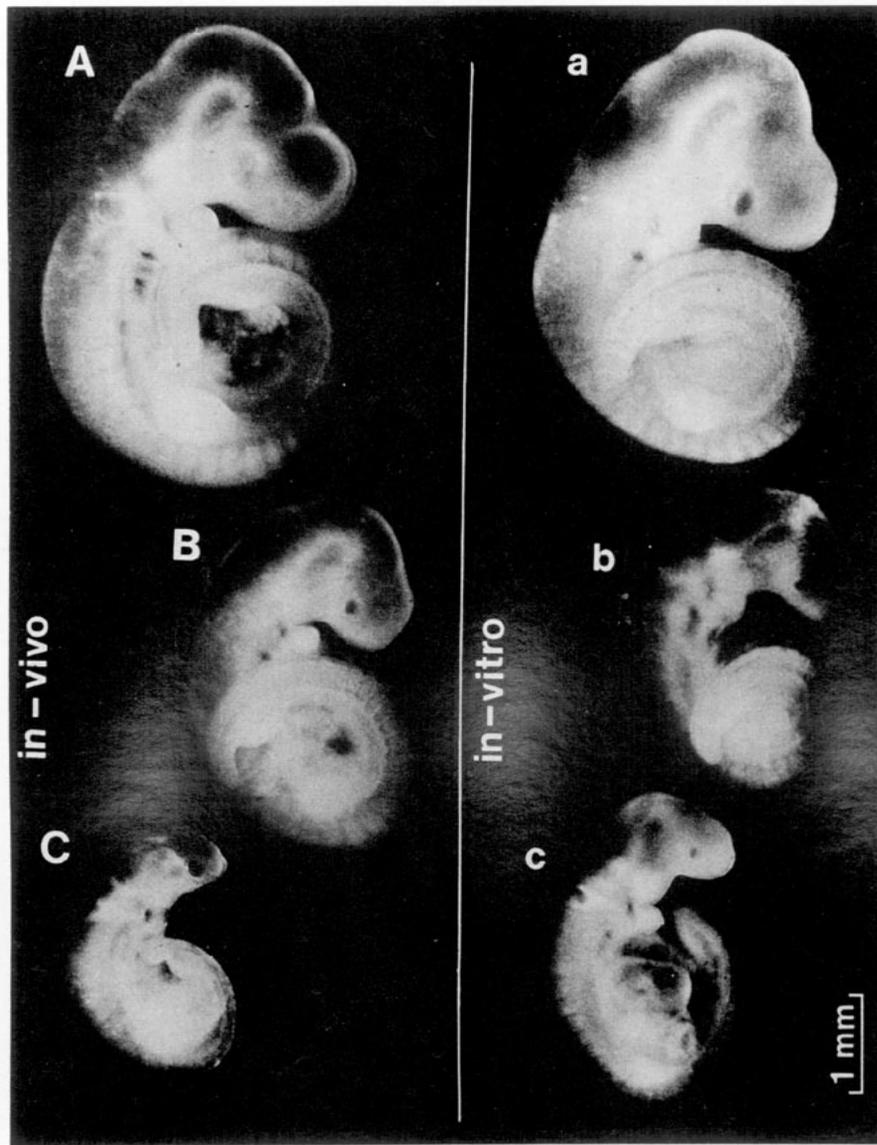


FIGURE 13. Effect of acyclovir on 11.5-day-old rat embryos *in vivo* and *in vitro* after 48-hr culture: (A) *in vivo* control; (B) 3×100 mg/kg acyclovir (treatment on day 10 of gestation); (C) 8×100 mg/kg acyclovir (treatment on days 9, 10, and 11 of gestation); (a) *in vitro* control; (b) $+ 100 \mu\text{M}$ acyclovir; (c) $+ 100 \mu\text{M}$ acyclovir.

Table 16. Gross structural abnormalities produced by acyclovir in rats *in vivo*.^a

Acyclovir treatment		Evaluation on day 11.5				
Day of pregnancy	Dose, mg/kg	Som ^b	Prot ^b	Score	ABN, %	
Control	$n = 22$	—	26.5	353	40	0
			26	299	40	
			25	244	40	
9, 10, 11	$n = 23$	8×50	27	—	40	3 % head
			26		40	
			25		40	
10	$n = 13$	1×200	25	158	35	100 % head
			24	135	33	
			24	114	33	

^aThe effect was evaluated on day 11.5 of pregnancy; acyclovir was given SC.

^bSOM = number of somites; Prot = $\mu\text{g}/\text{protein}/\text{embryo}$; ABN = abnormal embryos.

explanations appeared possible: (a) the substance does not reach the embryo *in vivo* at high enough concentrations to exert an embryotoxic potency, or, more likely, (b) the dose regime routinely applied was not adequate to allow the demonstration of an effect. Since the period of highest susceptibility could be deduced from our *in vitro* studies (day 10 of gestation), we applied the substance at a high dose during one day of pregnancy at the presumed susceptible period (Table 16). Surprisingly, the same type of structural abnormality was found under these special conditions *in vivo* when evaluated on day 11.5 of pregnancy as was seen *in vitro*. Figure 13 shows a typical picture of the embryos after treatment *in vitro* and *in vivo*.

To our knowledge this is the first example of an effect which, after being observed *in vitro*, has been predicted to also have a potential of occurring *in vivo* and subsequently has been demonstrated to be inducible *in vivo*. We feel that this gives a good example of the benefits of combining *in vitro* and *in vivo* tests for the assessment of a possible embryotoxic potential of a chemical.

These studies were supported by grants awarded to the Sfb 29 (special research group) and recently to Sfb 174 at the Freie Universität Berlin by the Deutsche Forschungsgemeinschaft. Ursula Bluth is acknowledged for her expert technical assistance and Ursula Schwikowski for the photographic work. We also thank Jane Klein-Friedrich for her help in preparing the manuscript.

Some of the data presented in this paper are parts of dissertations to be submitted to the Free University of Berlin.

REFERENCES

- Neubert, D. The use of culture techniques in studies on prenatal toxicity. In: *Pharmacology and Therapeutics*, Vol. 18 (J. G. Papp, Ed.), Pergamon Press, Oxford, 1982, pp. 397-434.
- Neubert, D. Benefits and limits of model systems in developmental biology and toxicology (*in vitro* techniques). In: *Prevention of Physical and Mental Congenital Defects*, Part A (M. Marois et al., Eds.), Alan R. Liss Inc., New York, 1985, pp. 91-96.
- Neubert, D. Toxicity studies with cellular models of differentiation. *Xenobiotica* 15: 649-660 (1985).
- Neubert, D., Blankenburg, G., Lewandowski, C., and Klug, S. Misinterpretations of results and creation of "artifacts" in studies on developmental toxicity using systems simpler than *in vivo* systems. In: *Developmental Mechanisms: Normal and Abnormal*. Alan R. Liss Inc., New York, 1985, pp. 241-266.
- New, D. A. T. Whole embryo culture and the study of mammalian embryos during organogenesis. *Biol. Rev.* 53: 81-122 (1978).
- Klug, S., Lewandowski, C., and Neubert, D. Modification and standardization of the culture of early postimplantation embryos for toxicological studies. *Arch. Toxicol.* 58: 84-88 (1985).
- Kao, J., Brown, N. A., Schmid, B., Goulding, E. H., and Fabro, S. Teratogenicity of valproic acid: *in vivo* and *in vitro* investigations. *Teratogen. Carcinogen. Mutagen.* 1: 367-382 (1981).
- Brown, N. A. Teratogenicity of carboxylic acids: distribution studies in whole embryos culture. In: *Drug Disposition in Teratogenesis* (H. Nau and W. J. Scott, Jr., Eds.), CRC Press, in press.
- Klug, S., Lewandowski, C., Nau, H., and Neubert, D. Teratogenicity of valproic acid and some of its metabolites in a whole embryo culture using bovine serum. ETS, 12th Conf., Sept. 1984, Veldhoven, Netherlands.
- Neubert, D., and Chahoud, I. Significance of species and strain differences in pre- and perinatal toxicology. *Acta Histochem.* 31: 23-35 (1985).
- Blankenburg, G. Some methods used for culturing embryonic tissues. Part B, Limb bud organ culture. In: *Culture Techniques* (D. Neubert and H.-J. Merker, Eds.), Walter de Gruyter Verlag, Berlin-New York, 1981, pp. 590-593.
- Barrach, H.-J., and Neubert, D. Significance of organ culture techniques for evaluation of prenatal toxicity. *Arch. Toxicol.* 45: 161-187 (1980).
- Nora, J. J., and Nora, A. H. Birth defects and oral contraceptives. *Lancet* i: 941-942 (1973).
- Nora, J. J., Nora, A. H., Blu, J., Ingram, J., Fountain, A., Peterson, M., Lortscher, R. H., and Kimberling, W. J. Exogenous progestogen and estrogen implicated in birth defects. *J. Am. Med. Assoc.* 240: 837-843 (1978).
- Levy, E. P., Cohen, A., and Fraser, F. C. Hormone treatment during pregnancy and congenital heart defects. *Lancet* i: 611 (1973).
- Harlap, S., Prywes, R., and Davies, A. M. Birth defects and oestrogens and progesterones in pregnancy. *Lancet* i: 682-683 (1975).
- Heinonen, O. P., Slone, D., and Shapiro, S. (Eds.). *Birth Defects and Drugs in Pregnancy*. Publ. Sci. Group Inc., Littleton, MA, 1977.
- Wiseman, R. A., and Dodds-Smith, I. C. Cardiovascular birth defects and antenatal exposure to female sex hormones: a re-evaluation of some base data. *Teratology* 30: 359-370 (1984).
- Davis, L. A., Sadler, T. W., and Langman, J. *In vitro* development of the heart under influence of retinoic acid. In: *Culture Techniques* (D. Neubert and H.-J. Merker, Eds.), Walter de Gruyter, Berlin-New York, 1981, pp. 101-115.
- Schardein, J. L. Congenital abnormalities and hormones during pregnancy: a clinical review. *Teratology* 22: 251-270 (1980).
- Wilson, J. G., and Brent, R. L. Are female sex hormones teratogenic? *Am. J. Obstet. Gynecol.* 141: 567-580 (1981).
- Davis, A., Krowke, R., Günther, T., Merker, H.-J., Neubert, D., Norppa, H., Sorsa, M., Vogel, E., Knaap, A. G. A. C., Voogel, C. E., van der Heijden, C. A., Kuroki, T., Umeda, M., Barale Loprieno, N., Malaveille, C., Brun, G., and Bartsch, H. Evaluation of the genetic and embryotoxic effects of bis[tri-*n*-butyltin]oxide TBTO, a broad spectrum pesticide in multiple *in vivo* and *in vitro* short-term tests. *Mutat. Res.*, in press.
- Platzek, T., Bochert, G., Schneider, W., and Neubert, D. Embryotoxicity induced by alkylating agents: 1. Ethylmethanesulfonate as a teratogen in mice. *Arch. Toxicol.* 51: 1-25 (1982).
- Krowke, R., Bluth, U., and Neubert, D. *In vitro* studies on the embryotoxic potential of bis[tri-*n*-butyltin]oxide in a limb bud organ culture system. *Arch. Toxicol.* 58: 125-129 (1986).
- Manson, J. M., and Simons, R. *In vitro* metabolism of cyclophosphamide in limb bud culture. *Teratology* 19: 149-158 (1979).
- Neubert, D., and Tapken, S. Some data on the identification of monooxygenases in fetal and neonatal mouse tissues. In: *Role of Pharmacokinetics in Prenatal and Perinatal Toxicology* (D. Neubert, H.-J. Merker, H. Nau, and J. Langman, Eds.), Georg Thieme Publ., Stuttgart, 1978, pp. 69-76.
- Nau, H., and Gansau, C. Development of cytochrome P-450-dependent drug metabolizing enzyme activities in mouse and human tissues *in vitro*. In: *Culture Techniques* (D. Neubert and H.-J. Merker, Eds.), Walter de Gruyter, Berlin-New York, 1981, pp. 495-507.
- Neubert, D., and Bluth, U. Limb bud organ cultures from mouse embryos after apparent induction of monooxygenases in utero; effects of cyclophosphamide, dimethylnitrosamine and some thalidomide derivatives. In: *Culture Techniques* (D. Neubert and H.-J. Merker, Eds.), Walter de Gruyter, Berlin-New York, 1981, pp. 175-195.
- Neubert, D., and Krowke, R. Effect of thalidomide-derivatives on limb development in culture. In: *Limb Development and Regeneration*, Part A. Alan R. Liss Inc., New York, 1983, pp. 387-397.
- Kastner, M., Blankenburg, G., and Schulz, T. Incorporation of an isolated and reconstituted cytochrome P-450 complex in an organ culture system. In: *Drug Disposition in Teratogenesis* (H. Nau and W. J. Scott, Jr., Eds.), CRC Press, in press.
- Neubert, D., Heger, W., Bremer, D., Frankus, E., Helm, F.-

- Ch., and Merker, H.-J. Some studies on the arene oxide hypothesis of the teratogenic action of thalidomide. In: *Drug Disposition in Teratogenesis* (H. Nau and W. J. Scott, Jr., Eds.), CRC Press, in press.
32. Nau, H., and Neubert, D. Development of drug-metabolizing monooxygenase systems in various mammalian species including man. Its significance for transplacental toxicity. In: *Role of Pharmacokinetics in Prenatal and Perinatal Toxicology* (D. Neubert, H.-J. Merker, H. Nau, and J. Langman, Eds.), Georg Thieme Publ., Stuttgart, 1978, pp. 13-44.
33. Moore, H. L., Jr., Szczech, G. M., Rodwell, D. E., Kapp, R. W., Jr., de Miranda, P., Tucker, W. E., Jr., Preclinical toxicology studies with acyclovir: teratologic, reproductive and neonatal tests. *Fund. Appl. Toxicol.* 3: 560-568 (1983).
34. Klug, S., Lewandowski, C., and Blankenburg, G. Effect of acyclovir on mammalian embryonic development in culture. *Arch. Toxicol.* 58: 84-88 (1985).