Methods for Estimating Risk of Chemical Injury: Human and Non-human Biota and Ecosystems
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Quantitative Dose–Response Models in Prenatal Toxicology

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ABSTRACT

Malformations and other embryotoxic effects are known to occur both in experimental animals and in man. However, not all substances unmasked in animals could also be proven to be effective in man. This does not necessarily imply a higher sensitivity of experimental species as compared to man, but rather different regions of the dose–response curves investigated. Attempts to reliably achieve quantification of embryotoxic effects have to take into account that:

—a no-observed-effect-level is most likely related to a true threshold below which no adverse effect occurs;
—defects detected in experimental animals reflect an embryotoxic potential; the defect does not have to occur in man;
—the condition leading to adverse effects must be given in detail such as: sensitive developmental stage(s), power of embryotoxic potential, dose-dependency of embryotoxic effects, metabolism including pharmacokinetics/toxicokinetics, and modes of embryotoxic action. Results of such properties tested in animal/man, and mother/fetus are valid aids for establishing quantitative risk assessment;
—dose–response relationships have not yet been established using epidemiological studies;
—the minimum requirements for successful quantitative risk estimation are the establishment of dose–response relationship in animals and comparative evaluation of pharmacokinetic/toxicokinetic behaviour in both species, animals and man.

1 INTRODUCTION

It is generally accepted that there are at least four types of non-heritable lesions which can be induced prenatally:

—structural abnormalities (teratogenic defects)

—lethal effects (embryo- or feto-mortality)
—growth retardation
—functional anomalies with only postnatal manifestation of defects (including prenatal or transplacental carcinogenesis).

All of them can be described as embryo- or fetotoxic effects—depending on the time of induction. The term 'prenatal toxicology' has been adopted in agreement with the terminology used in other fields of toxicological research and will be used throughout this paper (Bass *et al.*, 1977; Neubert *et al.*, 1978, 1980).

Among irreversible lesions, so-called gross malformations have been of greatest interest for a long time. Today we know that minor anomalies (i.e. those which are close to physiological variability) constitute a large proportion of teratogenic effects and often occur together with growth retardation of the whole organism in so-called syndromes (for example, ethanol, antiepileptics). Functional anomalies with postnatal manifestations have been recognized as part of such syndromes. Deviation from normal development of the central nervous system, including impairment of intelligence, seems to be of special significance.

From the many facets of teratology, i.e. prenatal toxicology, it becomes apparent that dose–response models in teratogenesis cannot include all aspects of quantitation at the same time. Investigations will have to focus on the assessment, for example, of the incidence rate of a given defect, or the possibility to increase the occurrence of different types of defects with increasing doses, or on dose–response comparisons of similar or analogue substances, or on the possible relationship between reversible and irreversible defects or between dose and sensitive stage of development. Examples of some of these aspects will be discussed.

2 TERATOGENIC POTENTIAL OF CHEMICAL SUBSTANCES: SPECIAL DETERMINANTS OF THE OUTCOME

According to the current concepts of toxicology, it should be possible to induce at least some kind of toxic action by treatment with any chemical substance, if it is applied at a high enough dose to the right species in the right way at the right time. The same is expected for embryotoxic effects, although evidence is being accumulated which shows that not all kinds of embryotoxic effects may be induced, even with extremely high doses of chemicals. Teratogenic effects may only be inducible in mammals with certain chemicals and not with others. We know from experience that only some of the substances experimentally defined as embryotoxic exhibit the same or similar properties in man. In fact, only a few chemical substances have so far been recognized as human teratogens. This is not so surprising since humans might not receive the dose required to induce embryotoxic effects. A similar situation is known to exist with carcinogens.

It seems that embryotoxicity follows the rule of thresholds applicable to most types of toxicity, for example acute toxicity, and practically all kinds of organ toxicity. Teratogens seem to behave in a different way than carcinogens or mutagens for which there seems to be no obvious threshold of action.

As a first step in assessing embryotoxicity of chemicals, a qualitative ('yes'–'no') evaluation should be attempted. At this stage, we should like to answer the question of whether a substance possesses an embryotoxic (teratogenic, embryolethal, etc.) potential. This requires taking into consideration the experimental conditions under which the embryotoxic effect has been observed. These conditions can often be clearly defined. (This is essential when attempting to make the next step in risk assessment: quantification of embryotoxic effects). Several qualifiers are used in the planning and interpretation of studies on dose–response relationships including the knowledge of:

(1) developmental stage(s) at which the embryotoxic effects can be induced;
(2) the kind of embryotoxic effect induced, and the pattern of abnormalities;
(3) the dose range in which the effect occurs;
(4) the species in which the effect occurs.

It can be helpful to have some understanding of possible mode of embryotoxic action.

By using such qualifiers in dose–response evaluation, the error margin inherent in the classification of chemical substances will become smaller.

2.1 Mutagenic Versus Embryotoxic Causes of Abnormalities

Preconceptional mutagenic effects on parental germ cells lead to random changes of the genome. Such inheritable gene defects can express themselves at critical stages of development, interfere with normal embryonic development, and, in some instances, lead to malformations. Their bearers often cannot give birth to a viable newborn and pregnancies result in dead embryos or fetuses. A mutagenic effect (spontaneous or induced by chemicals) rarely results in an increased occurrence of a certain specific type of malformation but rather in an increased background level of all possibly genetic abnormalities.

Postconceptional, prenatal, somatic mutations are of questionable significance for the induction of malformations. Non-heritable congenital abnormalities are induced by interference with the differentiation processes during prenatal development. They result from impairment by chemical substances or radiation of typical reactions necessary for normal development. Induced embryotoxic effects often include well-defined structural abnormalities compatible with life. Unlike mutagens, teratogens are known to produce specific malformations at high frequency. In animal experiments, the incidence of an abnormal development can be up to 100%, if the developmental stage at the treatment and the dose of the chemical are appropriate.

2.2 Inducibility of Defects in Relation to Developmental Stages

It is known that gross malformations can usually be induced only during organogenesis, whereas other types of embryotoxic effects (mortality, growth retardation) can also be induced before or after this developmental period. Certain malformations will usually occur after treatment with chemical substances during a part of organogenesis, resulting in phase-specific reactions of the developing organ.

But it must be stressed that many teratogenic agents, even when applied at the same stage of pregnancy, do not produce the same abnormality pattern, even when tested in the same species. Phase specificity, therefore, is a prerequisite, but in the majority of cases not all the possible abnormalities are induced by a given agent (cf. section 2.3.1).

The recognition of the principle that various types of abnormalities may be induced with largely varying susceptibilities and at different stages of prenatal development has led to a recommendation to treat the experimental animals over a period of at least 10 days (i.e., normal segment II study). This may have an advantage in the general analysis of the embryotoxic potential of a chemical, but it is less suitable for establishing dose–response relationships. For the latter studies, single applications of various doses are generally preferable. Treatment over extended periods of prenatal development often favours an embryolethal rather than a teratogenic effect. The treatment is given at stages of varying susceptibility and evaluated simultaneously; the embryotoxic effects tend to superimpose on each other. Therefore, quantification experiments and more sophisticated dose–response studies in general cannot be performed as the first step of testing but only in a subsequent experiment.

2.3 Modes of Embryotoxic Action

Our understanding of how a complete mammalian organism develops from a fertilized egg cell is not sufficient and, therefore, explanations on how toxicity is exerted during this development are almost non-existent; they are available only for some classes of compounds. The embryo or fetus is distinguished from other mammalian tissues by its very rapid rate of proliferation and a great variety of differentiation processes. We favour the assumption that an interference with differentiation processes (at the cellular or morphogenic level) is the preferred mode of action of agents with a teratogenic potential, as discussed in detail elsewhere (Neubert *et al.*, 1980). In this context, it is difficult to understand that 'unspecific' interference with DNA replication can also influence embryonic development via inhibition of proliferation. But it is possible that the inhibition of proliferation does not occur uniformly throughout the whole embryo.

2.3.1 Specific and Universal Modes of Teratogenic Action

Two modes of teratogenic action are revealed in the experimental and clinical literature:

—specific mode of teratogenic action, i.e. restriction to the induction of a circumscript and characteristic abnormality or pattern of abnormalities. Examples are available for drugs (thalidomide), hormones (glucocorticoids—cleft palate in rodents), and other chemicals (TCDD—cleft palate in mice). Even by increasing doses up to those that are toxic to the mother or by treating through one, several, or many days of gestation, this restricted pattern is not changed.

—universal mode of teratogenic action, i.e. a variety of different abnormalities is induced if the agent is applied at different stages of development. Multiple abnormalities are usually induced by X-rays, alkylating agents or anti-metabolites.

2.3.2 Modes of Action as Influenced by Phase and Dose-specific Reactions

A potential teratogen, be it 'specific' or 'universal' by its nature of action, can lead to the occurrence of a certain malformation only during a more or less limited phase of the gestation period. In addition to this phase specificity each embryotoxic agent induces a typical pattern of abnormalities ('drug specificity') and has its own dose–response relationship. By varying the doses applied, new malformations or even new types of embryotoxicity, such as teratogenicity and embryolethality, may occur ('dose specificity'), again governed by the 'specific' or 'universal' potential of the investigated compound. The bias exerted by other factors such as chemical specificity and species specificity will be dealt with later.

2.4 Power of Embryotoxic Potential

2.4.1 Rate of Expression of Embryotoxic Potential

Both from experimental research and from observations in man, it is known that the rate of expression changes with the substance investigated.

We can visualize a teratogenic substance which is able to induce gross abnormalities with a high degree of predictability, i.e. both its teratogenic potential and action are strong.

In contrast, we can often see that a compound induces minor abnormalities. Depending on the incidence rate (in many or only few litters) and on the repetitiveness of the pattern (similar or varying 'selection' from abnormalities recognized for the substance investigated), or on the increased occurrence of only one abnormality (major or minor, with higher or lower background), the teratogenic potential may be interpreted as weak, questionable, or non-existing.

2.4.2 Degree of Susceptibility of Embryonic Tissues

The relative embryotoxic (teratogenic) potency is not only defined by the expression rate of the damage in question. A variety of factors are increasingly being used to compare embryonic susceptibility (teratogenicity, embryolethality) with other characteristics (maternal lethality, other signs of maternal toxicity), in order to describe the relative potency by embryotoxic indices (Fabro *et al.*, 1982; Platzek *et al.*, 1982). Depending on the effects exerted at different levels (for example, molecular mode of action, morphological malformations), these factors or indices can be determined for the experimental species under investigation. A powerful, potent embryotoxic agent can thereby be defined independently of exposure level (an example will be presented in section 6.1).

3 EXPERIMENTAL INVESTIGATIONS OF QUANTITATIVE DOSE–RESPONSE MODELS

Quantification of results from animal experiments must be attempted by establishing dose–response relationships. In principle, the rules of pharmacology also apply in prenatal toxicology:

—the extent of manifestation of an effect is dose-dependent. This holds for the individual involved as well for the frequency observed in a population;
—the dose response for each embryotoxic effect has to be evaluated separately;
—increase of the dose may lead to additional effects, the action can no longer be confined to only a few organ systems;
—effects produced by very high doses may represent the sum of different embryotoxic actions.

Low doses of a teratogenic or embryotoxic substance often induce a defined abnormality, the frequency of occurrence being dose-dependent. At high doses the specificity is often lost and other abnormalities may occur with a whole variety of effects. At still higher doses, several types of defects are seen, for example, functional defects and malformations, retardation and malformations, malformations and lethal effects. At these high doses, the frequency of embryolethal effects may be raised to more than 30–50%, and a reliable evaluation of the extent of structural abnormalities becomes impossible.

Establishment of dose–response models is helpful for understanding the mode of embryotoxic action, species similarities or dissimilarities, quantitative comparison of different types of defects and the definition of threshold doses. However, for proper toxicological risk evaluation, information not only on dose–response relationships but also on pharmaco-/toxicokinetics and, if possible, on pharmaco-/toxicodynamics must be available. The evaluation, even of a clear-cut 'teratogenic' substance, may be difficult because of genetic modification of susceptibility in different strains and species.

3.1 Difficulties in Establishing Dose–Response Models

It may be impossible to investigate the occurrence of malformations over an extended and desired dose range owing to simultaneous occurrence of significant embryolethality in the same litter. Such a situation has been described, for example, for cyclophosphamide, for which lethality at higher doses finally masks the teratogenic effects (Bass *et al.*, 1979). Similarly, a very prominent structural defect (for example, a reduction anomaly) may cover more delicate defects seen at lower doses, again making the evaluation of dose–response relationships for a smaller defect difficult if not impossible. A known example is the induction of polydactyly with increasing frequency at higher doses, but higher doses of the same chemical induce phocomelia or even amelia as well (Neubert and Barrach, 1977). Under such conditions it may be worth considering grouping some types of abnormalities together (such as all limb defects) in order to obtain at least some possibility for establishing a dose–response relationship. This approach has been tried with some alkylating agents and it could be shown that, at least with this model, the dose–response curves for single and grouped abnormalities run almost parallel (Platzek *et al.*, 1982). But we should try to follow the principle, if at all possible, of evaluating single types of abnormalities in dose–response studies. Adding up various types of anomalies to one group in order to arrive at a quantification is unjustified until we have more knowledge on dose–response relationships in experimental teratology.

The experimental investigation of dose–response models is, of course, not restricted to structural abnormalities. Embryomortality may also be analysed in this way. This approach has been employed in studying the chemotherapeutic agents chloramphenicol and thiamphenicol. The embryolethal effect could be correlated with biochemical changes (Bass *et al.*, 1978c; see section 3.3).

3.2 Pharmacokinetics

Pharmacokinetic studies are helpful and often essential when attempting to quantify studies on embryotoxicity and to establish dose–response relationships, and when extrapolating toxicological data from one species to another. Application of, for example, chloramphenicol or thiamphenicol to pregnant rats and mice during organogenesis, shows these substances to be apparently of low toxicity for the embryo. But from pharmacokinetic studies it becomes clear that these drugs have a very short half-life in these rodents. By either repeated injections at 12-hour intervals (thiamphenicol), or continuous intravenous infusion (chloramphenicol), embryotoxicity in the form of prenatal mortality can be provoked, since under such experimental conditions drug concentrations can be achieved and maintained for sufficient periods of time to inhibit mitochondrial protein synthesis (see section 3.3). Under no conditions, even when applying these drugs over a wide dose range, could gross malformations be

detected (Bass, 1975). Doses needed to induce embryolethality in rats and mice differed by a factor of 2–3; both doses, however, appeared to be too high to be of any relevance to human intake of these drugs. After performing pharmacokinetic studies, it became apparent that lethality already occurred at low maternal serum and fetal tissue concentrations of thiamphenicol which were equal or lower than the concentrations of the drug which are used to treat infectious diseases in man (Bass *et al.*, 1978b). Such levels render mouse and rat fetuses equally susceptible as judged by prenatal mortality. The possibility of studying these drugs over a wide dose range (and no other type of embryotoxicity occurring at high doses) led to a dose–response model which could then be used to further investigate quantitative aspects that are also relevant to man.

3.3 Pharmacodynamics

When attempting to use quantitative dose–response models, the knowledge of the probable mode of embryotoxic action may be of advantage. This again can be demonstrated by the example of chloramphenicol/thiamphenicol. Similar to the inhibition of bacterial protein synthesis, mammalian mitochondrial protein synthesis is inhibited *in vitro* and *in vivo* by antibacterial concentrations of these drugs. This phenomenon has been studied in detail (Bass *et al.*, 1978c; Czempiel *et al.*, 1978, 1980). Although it applies to all types of tissue, toxic effects only occur in embryonic and some adult tissues. The rapid rate of proliferation typical for the stage of organogenesis is a prerequisite for the sensitivity of the embryo towards inhibition of mitochondrial protein synthesis (as in other rapidly proliferating tissues such as bone marrow). Since during the rapid growth the number of functioning mitochondria per cell is already kept low, further inhibition of mitochondrial protein synthesis by chemotherapeutic agents, such as chloramphenicol or thiamphenicol, makes energy supply in the form of mitochondrial ATP the rate-limiting step for embryonic development, and embryolethality results (Bass *et al.*, 1978a). Apparently, inhibition of energy generation during organogenesis constitutes such a general embryotoxic effect that no organ or tissue can be malformed. The result is an all or none effect leaving the *restitutio ad integrum* and prenatal mortality the only possibilities. Since it was proven that this toxicodynamic effect at the level of mitochondrial protein synthesis is the same in rodent and human tissues (Bass *et al.*, 1978c), the knowledge of the mode of embryolethal action aids extrapolation to the situation possibly existing in man.

3.4 Availability of Data

The amount of work required to gather sufficient data, and in the right proportions, for the analysis of dose–response relationships and the necessary

pharmacokinetic information may be substantial. Until now, useful data have been gathered and applied for the purpose of quantitative risk estimation for probably less than a dozen drugs and for very few environmental agents.

In routine studies, which normally provide the only type of data available for quantitative risk estimation, these problems and needs are almost never taken into account. The evaluation of a teratogenic potential seems possible for practical purposes with two to three teratogenic dose levels. It is obvious that these kind of studies can only give rough estimates of no-observed-effect level and mostly concentrate on the qualitative aspects of the problem. In addition to these restrictions another problem must be mentioned. While many of the more recent studies from industrial laboratories are performed under conditions which can form the basis for risk evaluation, this is not the case with the majority of data published from many university laboratories (experimental design, documentation of data, number of animals used, rigid control of experimental conditions, etc.).

4 QUANTIFICATION OF EFFECTS AS A MEANS FOR ESTABLISHING INTERSPECIES RELATIONSHIPS

An agent may give rise to different effects when tested in different species or even in different strains of the same experimental species. A classical example is thalidomide. Phocomelia and amelia as typical malformations seen in man can only be reproducibly induced in other primates. Different abnormalities from those seen in primates can be induced in rabbits and pigs. Rodent species in general do not show any typical abnormalities.

Methotrexate, a folate antagonist, is teratogenic in man (Wilson, 1977) and rats (Jordan *et al.*, 1977) at equivalent doses, whereas macaque monkeys seem to be surprisingly resistant (Scott *et al.*, 1978). Comparison of plasma concentrations in rats and monkeys was made by these authors, showing an appreciably higher capacity to clear this drug from maternal plasma in the monkey than in the rat. However, the difference in teratogenic susceptibility must apparently be attributed to other factors, i.e., greater inherent susceptibility of the rat since the embryolethality was induced in both species in spite of pharmacokinetic differences (Scott *et al.*, 1978).

From the information available today, it seems impossible, in the majority of cases, to predict the induction of a special type of abnormality in another species by trying to extrapolate findings of only one type of abnormality in the species experimentally studied. A specific embryotoxic effect can frequently be induced by a chemical only in one (or in a few) animal species while other species are apparently not susceptible. Among the many possible reasons for this species specificity, the following are probably the most important:

—Although the timing of application seems to aim at the same developmental stage, it is possible that some developmental processes are not alike or behave

in a different time-coordinated manner; or the known differences in the mode of placentation or type of histiotrophic nutrition play a decisive role.
—Differences in the maternal pharmacokinetics can cause critical concentrations in one species but not in another (see section 3.2).
—Differences in embryonic pharmacokinetics, such as the intrinsic capability to activate and/or inactivate a chemical substance can cause critical concentrations in fetuses of one species, but not in another; or the barriers between mother and fetus function differently or to a different extent.

In general, the same problems are encountered when extrapolating data from one animal species to another. Since most animal experiments are carried out in rodents, the extrapolation from rodents to man must take into account the possibility of special pharmaco- or toxicokinetic differences between rodent and man (Neubert *et al.*, 1978). If a chemical substance in unchanged form has an embryotoxic potential and reaches the embryo at a high enough concentration, it may be expected to exert, under suitable conditions, an embryotoxic effect in various species. If a chemical needs to be converted into an embryotoxic form, this may occur either in the maternal or the embryonic compartment. Only stable metabolites may reach the embryonic target at toxic concentrations. In order to act at the target cell, short-lived embryotoxic metabolites must be formed by the embryo or fetus itself; however, the inactive form must first pass to the fetus.

Especially in rodents, it seems to be difficult for the fetus to activate certain chemicals. This seems to be different in fetuses of primates. The pharmacologically and toxicologically important P450-type drug metabolizing enzyme system in human fetal liver is operative as early as in the 6th or 7th week of gestation (cf. Nau and Neubert, 1978). Little is known about the capacity of extrahepatic fetal tissue to metabolize xenobiotics. The information available indicates that during the embryonic period and the most significant phase of organogenesis the activity of mono-oxygenases is also low in primate embryos. Therefore, it is difficult to decide at present whether rodents represent good models for primates with respect to the ability to metabolize certain xenobiotics to active short-lived embryotoxic substances.

5 *IN VITRO* EXPERIMENTS AND CULTURE TECHNIQUES

During the last few years, several *in vitro* or culture techniques have been proposed for investigating prenatally induced toxic effects in mammals. Here, as in other fields where such or similar systems are employed, advantages and disadvantages are to be taken into account. A part of the complex situation, especially that which is governed by the maternal organism, can thereby be circumvented. For example, depending on the necessity to activate or metabolize a chemical into a more water-soluble compound, the absence of a maternal compartment may be a disadvantage. However, most of the arguments brought

forth against the use of culture techniques can also be used in favour of using them by choosing proper conditions. The absence of the placental barrier, easy control of the fetal drug concentration, easy variation of and access to nourishment, are just a few of the known arguments. Advantages and disadvantages have recently been discussed elsewhere in detail (Barrach and Neubert, 1980; Neubert, 1980, 1983; see these papers for further references). As to the quantification of effects, it is understandable that investigations on the mode of embryotoxic action as well as dose–response studies, can frequently be well performed by choosing the right culture and conditions. The study of the outcome of lesions developed in such systems may not be achieved so easily, however, since the whole gestation period cannot be covered by one culture system. This becomes especially apparent when postnatal observation of prenatally induced lesions is desirable.

6 QUANTITATIVE RISK ESTIMATION IN MAN

Quantitative risk estimation in man based on animal data is routinely attempted for pharmaceuticals; however, there are many limitations and problems (see sections 2 to 4). Usually qualitative risk assessment (i.e. the evaluation of the teratogenic potential with or without the qualifiers discussed in section 2) will be sufficient to allow the marketing or prohibiting of a drug. In doubtful cases, re-evaluation must be performed as soon as additional information from drug application in man becomes available. This additional information may change or confirm the previous decision.

In general, the same problems are encountered in quantitative risk assessment in man associated with exposure to environmental chemicals as they are in risk assessment for drugs. Data which are first available for the evaluation of chemicals will be almost exclusively animal data; data on prenatal toxicity in man are only seldom available. But under certain conditions a quantitative risk estimation is desirable. Ideally, we would like to be at the stage where this can be achieved for all chemicals.

Quantitative risk estimation in man must be discussed separately for the three theoretically possible data bases:

(1) animal data are the only source available for risk estimation;
(2) animal and human data are available for joint use in risk estimation;
(3) human data available overrule animal data obtained by standard protocol experiments.

6.1 Quantitative Risk Estimation Based on Non-human Data

Because of the limitations of quantitative risk estimation based solely on animal data, it becomes helpful or even essential to relate the effects investigated to additional variables which can be measured in the same experimental system.

The aim of looking for additional variables may be:

(1) either to better understand the dose–response relationship in question or to obtain information on possible thresholds, or
(2) to find a link which permits a better extrapolation of experimental findings from one species to another. Such a link could be pharmacokinetic information. (This also applies to risk estimation based on both human and non-human data; see section 6.2.)

Besides quantifying teratogenic effects as observed in experimental animals, it may be advantageous to obtain additional information on the mechanism of action of the teratogen. In this way it may become possible to draw more general conclusions on the relationship between the molecular reaction affected and the probability of an embryotoxic effect. This way facilitates risk evaluation. We have performed experiments with alkylating agents which are a particularly useful class of compounds for this purpose. They exert a variety of embryotoxic effects and are able to bind covalently to cell constituents. The dose–response relationship of this primary reaction can be measured. It has, therefore, been possible to establish a correlation between the teratogenesis and the biochemical event at the target tissues (the embryo). The molecular toxic event is assumed to be represented by the alkylation of the DNA of embryonic tissues.

The embryotoxicity of several directly acting alkylating agents has been investigated (methyl methanesulphonate and methyl nitrosourea, Bochert *et al.*, 1978; ethyl methanesulphonate, Platzek *et al.*, 1982). DNA alkylation experiments were performed using doses which had been shown to be effective in teratogenicity tests. The alkylation rate, expressed as pmole alkylated base per μmole guanine within the embryonic DNA, increases linearly with dose. A comparison of different substances showed great differences in the overall alkylation rate represented by the alkylation of N^7-guanine. But the alkylation rate of O^6-guanine correlated well within a range of 3–20 pmole/μmole with the dose of any alkylating agent able to trigger a teratogenic effect. Dose–response models were established by probit-analysis, and dose–response curves were computed using the maximum likelihood estimation. Effective doses were calculated and a teratogenic dose range (ED_{10} to ED_{90}) was defined for each substance. Further toxicological characteristics (maternal lethality, fetal and maternal weights) were additionally evaluated.

This approach has enabled us to predict from biochemical data the teratogenic potency of an agent from this class of chemicals and it also forms a sound basis for combination experiments.

6.2 Quantitative Risk Estimation Based on Non-human and Human Data

In some cases, because human data became available, the quantification of animal data attempted earlier had to be adjusted. From previous experiments

with pharmaceuticals we know that the time sequence of events can be quite variable.

Interspecies extrapolation from animals to man, or from man to animals, requires an answer to three questions:

(1) Is the same mode of toxic action to be expected in both species?
(2) Is the same or a similar embryotoxic effect to be expected in both man and experimental species?
(3) Which dose or dosing regimens may be expected to induce the same embryotoxic effect or to yield the same concentrations at the target in both species?

Question (3) can best be answered when the pharmacodynamics is known for both humans and experimental species used. This will be demonstrated with two examples: thiamphenicol and clonidine.

6.2.1 Risk Evaluation for Thiamphenicol

At the first sight, drugs such as chloramphenicol and thiamphenicol seem to be well suited for the treatment of maternal or fetal infections. No malformations have been reported to occur in experimental animals and, with the usual routine protocol for the evaluation of an embryotoxic potential in, for example, rats, only at extremely high doses could an embryolethal effect be observed. Therapeutic use has so far shown no increase in human malformations. However, comparative pharmacokinetic studies have revealed that the situation is more complex and it is now our opinion that, on the basis of the present knowledge, a human embryolethal effect cannot be ruled out.

In humans, including fetuses from therapeutic abortions, the $t_{1/2}$ is much longer than that determined in rats (3.5 compared with 1.5 hours). When considering this quantitative discrepancy and experimentally adjusting the dose and the treatment schedule in rats so that concentrations comparable to those used for therapeutic purposes are achieved in man (pharmacokinetics), the result was 100% embryolethality. The influence of thiamphenicol on human embryonic mitochondrial function (pharmacodynamics) is comparable to that studied in the rat (Bass *et al.*, 1978a,b,c). Since under these circumstances the animal model seems largely to mimic the situation to be expected in humans, we must arrive at the conclusion that thiamphenicol at therapeutic doses has an embryotoxic potency for man as well.

6.2.2 Risk Evaluation for Clonidine

Whereas the antihypersensitive and antimigraine drug clonidine indicated no sign of teratogenic potential in several species (up to 2000 μg/kg in rat, mouse and rabbit), several studies showed, although inconsistently and in most cases

without a clear-cut dose–response relationship, embryo- or fetolethal effects at pharmacologically active doses (sedation, piloerection, exophthalmus, etc.) that are only marginally toxic to the mother.

As outlined earlier, embryolethal effects and/or growth retardation can result from the application of maternally toxic doses of almost any chemical. This means that they can be signs not only of direct embryotoxicity, but also of maternal toxicity. According to our present knowledge, a teratogenic potential, however, is very unlikely to be provoked by maternal toxicity. If there are signs of maternal toxicity, embryolethal effects and growth retardation are qualified as unspecific and are usually of no further interest.

Only a close analysis of data will allow meaningful conclusions in such a case since spontaneous resorptions can occur in up to 10% of implantations in experimental animals; and spontaneous differences in the implantation rates per experimental series are common in rats, rabbits and mice. These two variables can lead to an apparent increase in resorption rate, masked by higher implantation rate giving an apparently normal number of live fetuses when compared with controls. Vice versa, the number of live fetuses can be apparently reduced without changing the percentage of resorptions if the average number of implantations in the experimental group is by chance lower than that in the control group. For borderline effects, conclusive evidence for an increased prenatal mortality should be considered if both an increase in resorption rate and a decrease in the number of live fetuses are found.

According to these criteria, one mouse experiment had to be judged positive at the highest dose of 2000 μg/kg but absolutely negative at 500 μg/kg. Three rat experiments at the highest dose of 150 μg/kg also showed a small increase in resorption rate, lower doses of 70, or 15 μg/kg respectively, gave negative results. The increased embryomortality was not accompanied by other signs of embryotoxicity such as growth retardation. In rats, the no-observed-effect level (NOEL), therefore, would be expected to be about 70 μg/kg (in mice much higher) after single daily applications.

For clonidine, risk evaluation with relevance to man was attempted following investigations of its pharmacokinetic behaviour. Such a procedure involves three steps:

(1) The pharmacokinetics must be known for the laboratory species to be used and for man.
(2) The dosing regimen is adjusted in the animal species, in our case the rat, to match the pharmacokinetics in humans; and
(3) this dosing regimen, which gives a similar pharmacokinetics in the plasma of the two species, is used for assessing the embryotoxic potency of the drug.

Pharmacokinetic studies indicated that the half-lives of clonidine in the two species, man and rat, are rather different. In man, a maximal plasma concen-

tration (c_{max}) of 0.95 ng/ml was found following an oral dose (^{14}C-clonidine) of about 2.5 μg/kg. The half-life was about 20 hours. In the rat, a c_{max} of about 15 ng/ml was reached after a dose of 100 μg/kg with a half-life of about 10 hours. 'Segment II studies', using one dose per 24 hours, would lead to plasma concentration profiles in the rat which could hardly be compared with those in man.

It was therefore decided to apply the drug to rats at 8-hour intervals (from day 6 to day 15 of pregnancy). Doses of, for example, 3×40 μg/kg daily produced plasma concentrations in the rat which were three times higher than the c_{max} measured in humans (3×150 μg daily) and the c_{min} in humans was still exceeded in the rat by a factor of 2. Such plasma concentrations in the rat, which exceeded those known to occur in man 3-times (c_{max}) to 2-times (c_{min}), did not induce an embryotoxic effect. Those exceeding the concentrations by more than 6-times (c_{max}) and 4-times (c_{min}), respectively, were embryotoxic. As judged from these data, therapeutic doses of 3×150 μg daily are not expected to exert embryotoxic effects in man.

In both examples discussed, the amount of human data available did not allow a reliable risk assessment. Theoretical or practical suspicion, however, led to performing other animal experiments in addition to those required by the standard protocol. Although it was very tedious to obtain the necessary data, we showed that the pharmacokinetic approach (steps 1 to 3) was feasible, that the conclusions were useful and allowed classification of pharmaceuticals as 'probably embryotoxic' or 'safe for man'.

6.3 Embryotoxicity as a Relative Risk

In animal studies, the demonstration of a teratogenic potential (qualitative risk evaluation, 'yes' or 'no' answer) is only the first step (see Neubert, 1983). Quantification of the teratogenic effect (dose–response, NOEL) has to follow. But most important is the analysis of the potency of the embryotoxic effect when compared to maternal toxicity. If the substance is applied over an extended period of pregnancy (as it is done in all routine studies), the acute maternal toxicity (such as LD_{50}) is not a suitable measure with which to compare the embryotoxicity.

We have suggested (Platzek *et al.*, 1982) a procedure that may be followed in such studies. The teratogenic dose range was defined (ED_{10}–ED_{90}) and maternal toxicity measured. In analogy with the 'therapeutic index', the use of an 'embryotoxic index' was proposed, such as, the ratio of ED_{10} for maternal mortality and ED_{90} for teratogenicity. The quantification of the fetotoxic effects by their own 'indices' is a prerequisite for calculating the 'embryotoxic index' then used for comparison of the relative teratogenic potency of different compounds.

A similar approach was suggested by Fabro *et al.* (1982). A so-called 'relative

teratogenic index' was established by the ratio of LD_{01} (adult lethality) and ED_{05} (teratogenicity) for several anticonvulsants.

We expect that the introduction and use of these indices is a step forward in quantitative risk estimation based on animal data. However, the usefulness of such indices depends on choosing the correct parameters.

6.4 Quantitative Risk Assessment Based on Human Data: Risk Evaluation for Bendectin®

Bendectin® (doxylamine and pyridoxine) and Lenotan® (which in addition contains dicycloverine) have been used to relieve severe vomiting during pregnancy. During the last years, human data from approximately 10 000–15 000 mother–child pairs have been gathered. Published studies do not demonstrate the association between drug intake and increased risk of birth defects (Michaelis *et al.*, 1980; Cordero *et al.*, 1981; Fleming *et al.*, 1981; Jick *et al.*, 1981; Mitchell *et al.*, 1981; and other earlier or more recent publications). Since it is impossible to prove that a drug never causes malformations, even those pharmaceuticals well studied in man should be used with caution (Bass, 1979).

For Bendectin®, the risk assessment is based solely on interpretation of human data, setting aside data on dose–response relationships in rodents. Using only these data it has been concluded that the malformation risk, if any, is small. Such a strong statement would have never been allowed if based on animal data alone.

We think we have shown that the quantitative risk estimate can depend on both the amount and type of human and animal data available. Depending on the amount of information required and/or the available feedback from human data, additional especially designed animal studies can be performed.

7 DIFFERENT TYPES AND DEPTH OF INVESTIGATION

Data from the standard protocol experiments (so-called segment I, II, and III studies) are usually available today for drugs but seldom for other classes of chemicals. Chemicals at the workplace, for example, are regulated mostly through a classification of hazard resulting from acute exposure.

There are two philosophies available today which lead to quite different degrees of risk quantification. Both, however, depend on the availability of pharmacokinetic data (see also 3.3, 6.2.2).

7.1 Step by Step Comparison and Evaluation

(1) From pharmacokinetic data in the animal species chosen for investigation and from pharmacokinetic data in man (preferably including those in pregnant women and in embryo/fetus target) the plasma concentration to be obtained as equivalent of therapeutic doses is calculated;

(2) From this data a dosing regimen is derived which takes into account, for example, differences in $t\frac{1}{2}$ between animals and man, thus simulating human pharmacokinetic conditions in the animal;
(3) In addition to this low, therapeutic dosing regimen, two higher dosing regimens are used (the highest dose employed is expected to show some maternal toxicity, such as retarded increase of weight);
(4) From the results obtained, NOELs are defined and the safety margin for each type of defect calculated;
(5) The mode of teratogenic action may have to be determined in special experiments and systems (pharmacodynamics), including *in vitro* culture systems;
(6) From these experiments, special characteristics discriminating prenatal toxicity (sections 1 and 2) have to be defined for the chemical under investigation: specific/general and strong/weak defects occurring at doses high/low as compared with doses toxic to the mother;
(7) The safety margin available is related to the type of prenatal toxicity observed. The safety factors will tend to become smaller when the reliability of the available data increases. For strong specific teratogenic effects, there is no example available where a safety factor has been successfully calculated and used; for strong, universal teratogens, such as cytostatic agents, today the therapeutic requirement overrules the desire for safety.
(8) A dosing regimen, including the safety margin, showing no pathological findings, must then be called 'safe for man at the safety margin employed.'

7.2 Decision Approach

This scheme follows the first four steps of section 7.1. Then a decision is made depending on the occurrence of any embryotoxic effect but no differentiated evaluation of the various types of damage and their severity is attempted. This approach is based on the assumption that the occurrence of one type of defect makes more likely the occurrence of other types of defects which could not be observed under the limited test conditions. This decision leads to making the chemical available or not available to pregnant women, including some type of warning.

It has to be pointed out that whatever means of quantification of embryotoxicity is used, decisions are based on dose–response relationships of embryotoxic defects detected in animals. The dose–response relationships for possible damage usually cannot be established in women, be it by monitoring processes or by special directed epidemiological investigations. The data obtained from such studies may in a sense appear semiquantitative: high or low rates of incidence for different malformations to be related to different chemical substances. Most of the factors qualifying embryotoxicity discussed earlier cannot be evaluated from

human data. This kind of quantification is a domain of animal experiments, including *in vitro* culture studies.

The two approaches described, the step by step and the decision approach, may both be useful. For drugs with indicated use in human pregnancy, the step by step approach seems to be the correct way of risk evaluation. For other types of chemicals, a yes/no answer may be sufficient to approve (or not to approve) their use, thereby allowing for the quantification process to be performed in the second step.

8 REFERENCES

Barrach, H. J., and Neubert, D. (1980). Significance of organ culture techniques for evaluation of prenatal toxicity. *Archs Tox.*, **45,** 161–187.

Bass, R. (1975). Proposal of a mechanism for the induction of embryolethal effects. In Neubert, D., and Merker, H.-J. (Eds.) *New Approaches to the Evaluation of Abnormal Embryonic Development*, pp. 524–541. Georg Thieme Verlag, Stuttgart.

Bass, R. (1979). Antiemetika in der Früschwanngerschaft Münch. *Med. Wschr.*, **121,** 36–38.

Bass, R., Bochert, G., Merker, H.-J., and Neubert, D. (1977). Some aspects of teratogenesis and mutagenesis in mammalian embryos. *J. Tox. envir. Hlth*, **2,** 1353–1374.

Bass, R., Bordasch, K., and Merker, H.-J. (1979). Embryotoxic effect of cyclophosphamide: is it possible to differentiate between its teratogenic and embryolethal mode of action? In van Julsingha, E. B., Tesh, J. M., and Fara, G. M. (Eds.). *Advances in the Detection of Congenital Malformation*, pp. 198–202. Micheal Robbin, Printer, Chelmsford, Essex.

Bass, R., Oerter, D., Krowke, R., and Spielmann, H. (1978a). Embryonic development and mitochondrial function. III. Inhibition of respiration and ATP generation in rat embryos by thiamphenicol. *Teratology*, **18,** 93–102.

Bass, R., Schütte, I., and Nau, H. (1978b). Placental transfer and fetal distribution of thiamphenicol in pregnant rats and humans. In Neubert, D., Merker, H.-J., Nau, H., and Langman, J. (Eds.) *Role of Pharmacokinetics in Prenatal and Perinatal Toxicology*, pp. 507–518. Georg Thieme Verlag, Stuttgart.

Bass, R., Ulbrich, B., and Welsh, F. (1978c). Thiamphenicol: in vitro accumulation by human placenta and correlation with inhibition of mitochondrial protein synthesis in human and rat placental and fetal mitochondria. In Neubert, D., Merker, H.-J., Nau, H., and Langman, J. (Eds.) *Role of Pharmacokinetics in Prenatal and Perinatal Toxicology*, pp. 519–525. Georg Thieme Verlag, Stuttgart.

Bochert, G., Rahm, U., and Schnieders, B. (1978). Pharmacokinetics of embryotoxic direct-acting akylating agents: comparison of DNA alkylation of various maternal tissues and the embryo during organogenesis. In Neubert, D., Merker, H.-J., Nau, H., and Langman, J. (Eds.) *Role of Pharmacokinetics in Prenatal and Perinatal Toxicology. Third Symposium on Prenatal Development*, pp. 235–250. Georg Thieme Publishers, Stuttgart.

Cordero, J. F., Oakley, G. P., Greenberg, F., and James, L. M. (1981). Is Benedictin^{R} a teratogen? *J. Am. med. Ass.*, **245,** 2307–2310.

Czempiel, W., Ulbrich, B., and Bass, R. (1978). The effects of chloramphenicol and its analogues on 55 S, 70 S and 80 S ribosomes: the implication to their embryotoxic mode of action. In Neubert, D., Merker, H.-J., Nau, H., and Langman, J. (Eds.) *Role of*

Pharmacokinetics in Prenatal and Perinatal Toxicology, pp. 527–534. Georg Thieme Verlag, Stuttgart.

Czempiel, W., Ulbrich, B., and Bass, R. (1980). Mammalian mitochondrial ribosomes: studies on the exchangeability of polypeptide chain elongation factors from bacterial and mitochondrial systems. *Eur. J. Biochem.*, **108,** 337–343.

Fabro, S., Shull, G., and Brown, N. (1982). The relative teratogenic index and teratogenic potency: proposed components of developmental toxicity risk assessment. *Teratog. Carcinog. Mutag.*, **2,** 61–76.

Fleming, D. M., Know, J. D. E., and Crombie, D. L. (1981). Debendox in early pregnancy and fetal malformation. *Br. med. J.*, **283,** 99–101.

Jick, H., Holmes, L. B., Hunter, J. R., Madsen, S., and Stergachis, A. (1981). First-trimester drug use and congenital disorders. *J. Am. med. Ass.*, **246,** 343–346.

Jordan, R. L., Wilson, J. G., and Schumacher, H. J. (1977). Embryotoxicity of the folate antagonist methotrexate in rats, rabbits. *Teratology*, **15,** 73–80.

Michaelis, J., Glück, E., Michaelis, H., Koller, S., and Degenhardt, K. H. (1980). Teratogene Effekte von Lenotan? *Dt. Ärzteblatt*, **23,** 1527–1529.

Mitchell, A. A., Rosenberg, L., Shapiro, S., and Slone, D. (1981). Birth defects related to Bendectin® use in pregnancy. *J. Am. med. Ass.*, **245,** 2311–2314.

Nau, H., and Neubert, D. (1978). Development of drug-metabolizing monooxygenase systems in various mammalian species including man. Its significance for transplacental toxicity. In Neubert, D., Merker, H.-J., Nau, H., and Langman, J. (Eds.) *Role of Pharmacokinetics in Prenatal and Perinatal Toxicology. Third Symposium on Prenatal Development*, pp. 13–44. Georg Thieme Publishers, Stuttgart.

Neubert, D. (1980). Prospective in-vitro-Modelle als Ersatz für Langzeituntersuchungen. [Prospective in vitro models as a substitute for long-term tests.] In Schnieders, B., and Grosdanoff, P. (Eds.) *Zur Problematik von chronischen Toxizitätsprüfungen*, pp. 277–287. Dietrich Reimer Publisher, Berlin (in German).

Neubert, D. (1983). The use of culture techniques in studies on prenatal toxicity. In Papp, J. G. (Ed.) *Developmental Pharmacology, International Encylopedia of Pharmacology and Therapeutics*. Pergamon Press, Oxford (in press).

Neubert, D., and Barrach, H.-J. (1977). Techniques applicable to study morphogenetic differentiation of limb buds in organ culture. In Neubert, D., Merker, H.-J., and Kwasigroch, T. E. (Eds.) *Methods in Prenatal and Perinatal Toxicology. Evaluation of Embryotoxic Effects in Experimental Animals*, pp. 241–251. Georg Thieme Verlag, Stuttgart.

Neubert, D., Barrach, H.-J., and Merker, H.-J., (1980). Drug-induced damage to the embryo or fetus. (Molecular and multilateral approach to prenatal toxicology). In Grundmann, E. (Ed.) *Drug-Induced Pathology. Current Topics in Pathology*, Vol. 69, pp. 241–331. Springer-Verlag, Berlin, Heidelberg, New York.

Neubert, D., Hinz, N., Baumann, I., and Barrach, H.-J. (1978). Attempt upon a quantitative evaluation of the degree of differentiation or of the degree of interference with development in organ culture. In Neubert, D., Merker, H.-J., Nau, H., and Langman, J. (Eds.) *Role of Pharmacokinetics in Prenatal and Perinatal Toxicology*, pp. 213–228. Georg Thieme Verlag, Stuttgart.

Platzek, T., Bochert, G., Schneider, W., and Neubert, D. (1982). Embryotoxicity induced by alkylating agents: I. Ethylmethanesulfonate teratogen in mice—a model for dose-response relationships of alkylating agents. *Archs Tox.*, **51,** 1–25.

Scott, W. J. (1978). Cell death and reduced proliferation rate. In Wilson, J. G., and Fraser, F. C. (Eds.) *Handbook of Teratology*, Vol. 2, pp. 81–98. Plenum Press, New York, London.

Scott, W. J., Wilson, J. G., Ritter, E. J., and Fradkin, R. (1978). Further studies on

distribution of teratogenic drugs in pregnant rats and rhesus monkeys. In Neubert, D., Merker, H.-J., Nau, H., and Langman, J. (Eds.) *Role of Pharmacokinetics in Prenatal and Perinatal Toxicology*, pp. 499–503. Georg Thieme Verlag, Stuttgart.

Wilson, J. G. (1977). Embryotoxicity of drugs in man. In Wilson, J. G., and Fraser, F. C. (Eds.) *Handbook of Teratology*, Vol. 1, pp. 309–355. Plenum Press, New York, London.