Thresholds in Toxic, Teratogenic, Mutagenic, and Carcinogenic Effects

by Ernst Freese*

Many inhibitory and toxic effects show a dose dependence for which the extrapolation of a linear plot to zero or background effect cuts through the abscissa at some positive dose, indicating a threshold concentration below which the agent seems to be ineffective (*1*). If the teratogenic effect of such an agent is caused by the same cellular reaction, its dose–effect curve may also exhibit a threshold, though with a lower value. In contrast, for most mutagenic compounds a linear plot of the frequency of induced mutations against the dose (concentration) has exhibited no threshold; such experiments were done with transforming DNA or bacterial viruses (*2–4*), bacteria or fungi (*5, 6*), plants (*7–9*, Sparrow and Scheirer, personal communication), insects (*10*), and mammalian test systems (*11, 12*). An apparent threshold of mutation induction has been observed only rarely, usually where the investigated cells could be reached only after the mutagen had passed several barriers (*12, 13*). One may therefore ask whether there are fundamental reasons for this different dose response of toxic versus mutagenic effects and under which conditions a threshold effect could be expected. Such considerations are important for the experimental evaluation of the potential hazard of pesticides, drugs, or food additives.

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Before this problem is examined, a few words are needed concerning the extrapolation of experimental data. Such data can always be plotted in a number of ways, e.g., linearly against the dose, against some power of the dose, or against the logarithm of the dose (as is usually done for toxic compounds with very different potencies). To demonstrate its increase with some power of the dose, one can also plot the logarithm of the effect against the logarithm of the dose. While any of these plots can be used to display experimental points, an extrapolation of such points to lower dose values is justified only if there are good theoretical reasons to assume a particular dose dependence (linear, square, etc.). In many experimental systems, especially in simple microbial tests, there are good chemical reasons to assume that the effect at low doses increases linearly with (or with the square of) the dose, and the experimental data have verified this assumption wherever it was tested extensively (*2, 3*); a straight-line extrapolation of a linear plot (or a plot against the square of the dose) is then justified. However, if no such assumptions are warranted, the following approach seems the only one justified. After making as many measurements as feasible, one calculates for various possible dose–effect curves (e.g., polynomials with unknown coefficients and exponents), the best least-square approximation of all the experimental points (with different weights if warranted), and the mean-square deviation from the calculated

curve (such calculations are simple and fast if modern computer programs are used). At this stage, one can either assume that the curve with the lowest mean-square deviation represents the correct curve, or one can still insert a theoretical bias to select out of several curves with similar mean-square deviations the one which appears most likely correct. Although this approach is not as precise as obtaining more data, it certainly is more satisfactory than the guesses often used in the interpretation of experimental data. A thorough statistical evaluation of such data is the more valuable the more complex the biological system, because the time needed for analysis represents a progressively smaller fraction of the time needed to acquire additional data at lower dose levels. Unfortunately, experimental data are often too sparse to allow any statistical curve fitting.

Toxic Effects

Most toxic compounds or other agents in commercial or medical use inhibit cellular reactions reversibly, some inhibit irreversibly, and few kill individual cells.

Reversible inhibition may affect, e.g., an enzyme or the transport mechanism of the cell. It is subject to the mass action laws summarized by the Michaelis-Menton equation (*1*).

If the inhibited reaction is rate-limiting, the extent of inhibition I is described by:

$$I = \frac{c}{(c + k)} \qquad (1)$$

where c is the concentration of compound and k is a constant. When I is plotted against c, the curve increases linearly from the zero point and saturates at the value 1 (100% inhibition). When I is plotted against the logarithm of c, a sigmoid curve is obtained. Such semilogarithmic plots are frequently used for the comparison of drugs with different potency. (One might be tempted to extrapolate the curve through the experimental points of a semilogarithmic plot to some positive concentration value on the abscissa, especially since the statistical variation of values determined at lower drug concentrations is high. Thus one might erroneously conclude the existence of a threshold. As explained above, an extrapolation of such inhibition data is justified only for a linear plot of the experimental values.)

A true threshold of the observable biological effect may exist if the inhibited reaction does not normally limit the rate of cell metabolism or of an organ's function. In that case, no biological effect will be observed until the inhibitor concentration has exceeded a certain minimal (or threshold) value T. The inhibition equation then takes the form:

$$I = \frac{(c - T)}{(c + K)} \qquad (2)$$

Zero inhibition is obtained when $c = T$. The curves described by eq, (2) are the same as for eq, (1), except that they are shifted along the abscissa by the value T (if $K = k - T$).

For slowly reversible or irreversible inhibition the situation is similar, but the equations become more complex and in the case of irreversible inhibitors cannot be explained by the Michaelis-Menton theory (*1*). Again, a threshold value may exist if the inhibited reaction is not rate limiting.

A toxic effect may also result from the killing of a number of cells in a multicellular organism. Cell death is most generally defined by the inability of the cell to multiply. It can be caused by the cumulative effect of inhibitor molecules, leading to the leakage of the cell membrane, the activation or induction of a protease or nuclease, or the irreversible inactivation of an enzyme. Cell death caused by such changes can often be detected by the irreversible destruction of most cell functions or by the uptake of basic dyes (trypan blue). A cell's ability to multiply can also be destroyed by the irreversible reaction of molecules with DNA, leading to chromosome breakage or to other lethal modifications of the genetic material. Such changes can usually not be detected by functional tests of resting cells but only by

cytological means or by challenging the cell's ability to replicate.

In an adult organism, the death of a few cells usually has no observable effect, while death of many cells in an organ will show up as a toxic reaction. If the rate at which an organ normally functions is not limited by the number of its cells, a reduction of this rate may show up only when the number of killed cells exceeds a certain threshold value. Only a large extent of cell death may therefore be registered as a toxic effect. Different organs of the same organism are consequently affected to different degrees by the same frequency of cell death. Since different organisms, including different humans, differ in their genetic constitution, they also have different thresholds for organ failure or for death.

An apparent threshold can result if the compound can react with some cellular components or be destroyed by some enzyme before it reaches its target. A strong toxic effect may then be observed only when the concentration of the compound exceeds a critical value above which the number of reactive sites or the turnover of the destroying enzyme do not suffice to prevent the accumulation of the inhibitor. This critical value would not represent an absolute threshold, because the effectiveness of the drug would be only greatly reduced but not completely eliminated at lower inhibitor concentrations.

Teratogenic Effect

When a compound acts during early embryogenesis (organogenesis), while the anlagen for different organs are laid down, growth inhibition or death of a few cells can drastically affect the relative ratio of differentiated cells giving rise to an organ (*14*). A teratogenic effect can therefore be produced by an inhibitory or lethal reaction of so few cells that a toxic effect would not yet be detectable in an adult organism. Moreover, a low inhibitor concentration, which does not restrict the function of resting cells or adult organs, may already reduce the rate at which certain embryonic cells replicate. Similarly, a small number of killed cells, which does not limit the function of an organ, may already be disastrous for development. The threshold (if any) for teratogenic effects is presumably lower, therefore, than that for toxic effects.

Mutagenic Effect

A mutation can be induced by the reaction of one or two reagent molecules with DNA. Such reactions can produce base pair changes that lead to point mutations and alter the functional properties of single genes, or they can produce large chromosome alterations that affect several genes or even whole chromosome segments. Mutagenic effects are more insidious than toxic effects, for a single mutated cell that multiplies can produce a massive effect in an organism. In comparison, the death of a single cell is relatively harmless, except when it occurs during early embryogenesis. If the mutation affects a germinal cell, a dominant or recessive hereditary disease or loss of vitality may arise in the offspring; if it alters an embryonic cell a malformation may ensue; if it affects an adult cell, a tumor or leukemia may develop (*15*).

Most direct mutagens react covalently with DNA, are incorporated into DNA, or bind quasi-irreversibly to DNA or its synthase. All these reactions have been found to increase linearly with the concentration of the compound. There is apparently no intracellular process by which a mutation can be eliminated from a cell, once it has been finalized in both strands of the double-stranded DNA molecule.

If the mutated cell has an altered surface, it can be recognized as a strange body and be removed by phagocytosis or be coated by circulating antibodies; such surface recognition may in fact provide the major protection of higher organisms against a high incidence of malignant growth (*16*). Without such surface changes, however, the mutated cell can persist, and even with such changes a fraction of mutated cells apparently can occasionally replicate beyond the stage at which antibodies or other cellular

reactions can arrest further growth. In normal animals, more than one DNA alteration per cell may be required to cause some types of cancer, because the production of pulmonary tumors in mice increased approximately with the square of the concentration of different alkylating chemicals (*17*). The possibility that multiple mutations are required for cancer had earlier been proposed by Ashley (*18*). A statistical analysis of retinoblastomas in humans also indicated that two separate events (mutations) are needed for the occurrence of most monolateral cases; however, only one such event is required for bilateral cases, the other mutation apparently being inherited (*19*). Also, radiation-induced leukemia and tumors increased linearly with the dose (*20*). In any case, neither for radiation nor for carcinogens that can react directly with DNA has the dose dependence indicated the presence of a threshold (*17, 20, 21*).

Nevertheless, an apparent or a true threshold of mutation (or cancer) induction can be expected to occur for a variety of reasons. The frequency of such genetic effects in different tissues is affected by the rates of distribution, absorption, metabolism, and excretion of the mutagen, each of which can be influenced by genetic and environmental factors. For example, an apparent threshold concentration could be expected either if the mutagen would be very effectively destroyed before it could reach the nucleus (e.g., peroxides and other radical-producing compounds are destroyed by peroxidase or catalase) or if it would bind to, be taken up by, or otherwise react with the cells close to the site of administration, so that it could not reach the germinal cells whose mutation alone would be measured in certain tests. Only rather high concentrations of a compound might then show a strong mutagenic effect.

As another possibility, the activation or destruction of the mutagen may require enzyme reactions that can be induced either by the mutagen itself or by some other compound. Since the importance of such enzyme reactions (often microsomal enzymes) has been realized, the correlation between mutagens and carcinogens has steadily increased (*15, 22, 23*). The concentration or time dependence of induction might then create the impression of a threshold effect. The extent of repair mechanisms in a cell also influences the frequency of mutations; this frequency differs in genetically different organisms. Such repair mechanisms may be able to handle only a limited number of DNA alterations and be overwhelmed by too high concentrations of a mutagen, giving then rise to a vast increase in mutation frequency.

A true threshold effect could be expected for the few weak mutagens that inhibit some enzyme needed to produce a precursor of DNA; if that enzyme normally would not limit the rate of DNA or chromosome replication, the compound would be effective only if its concentration exceeded a minimal value.

Summary

The overall effect by which the reaction of chemical or physical agents with cells influences an organism depends on the type of reaction, the kind of cell, and the developmental stage of the organism. Two major factors have to be considered.

The inhibition or death of cells may have drastic consequences for an afflicted cell, but it influences an adult organism only if it occurs in many cells. Such massive reactions produce toxic effects or, if they occur in germ cells, cause sterility. Only during early development can the reactions of a few cells be disastrous for the whole organism, e.g., if they produce malformations (teratogenic effects). In contrast, the mutation of a single cell can always have drastic consequences for an organism, because if the mutated cell replicates, it can produce hereditary alterations, malformations, or cancer.

When the affected cellular reactions do not limit the rate at which the investigated organ functions, there will be a threshold concentration below which no functional defect is produced. Such a threshold phenomenon occurs frequently for inhibitory or lethal cell reactions, but both molecular con-

siderations and experimental results show that it is an exception for mutagenic alterations.

Conclusion

If one wants to protect mankind adequately against mutations, one should extrapolate mutagenic or carcinogenic effects, observed at high doses of a compound, either linearly or with some exponent of the concentration, if that can be established at high doses (concentrations), to the spontaneous background effect of zero dose, unless statistically significant measurements or knowledge of the mutagenic mechanisms warrant otherwise. There is certainly no justification to assume a positive no-effect (background only) dose for any mutagen or carcinogen, except when the molecular mechanism by which the particular compound induces mutations renders such a threshold effect likely (e.g., if the mutations are produced by enzyme inhibition). Such a postulate is necessary, because mutagenic or carcinogenic tests in mammals are expensive and time-consuming, so that it is not feasible to examine the mutagenic effect of many compounds down to the low concentrations to which mankind is exposed. It is also needed as a minimum safety valve, because the genetic and nutritional variation of people and the influence of other environmental compounds that act as activators or reducers of a mutagenic effect can greatly influence the mutagenicity or carcinogenicity of a given compound. Society may decide that the benefit of some compounds warrants a small risk whose level can be set at a certain value, as was done for x-radiation (*20*). Those who then still advocate the widespread use of a compound at a dose exceeding the accepted risk level, because they claim the existence of a threshold dose below which the compound might be genetically ineffective, should be obliged to prove their contention by statistically significant measurements of the genetic effects of the compound at the proposed human dose in all usually employed mammalian test systems.

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Discussion

Dr. R. B. Cummings (*ORNL*): I'd like to make a comment about EMS reaching the target; it does reach the target. If one looks at the ethylation of DNA in sperm cells plotted against the administered dose interperitoneally, the effect is linear with dose in a range from 400 to 50 mg/kg, and at 5 mg/kg it is only slightly off that linear extrapolation downward. If one looks at ethylations/nucleotide DNA extracted from the whole testes after the DNA is cleaned up on a cesium chloride gradient, again the ethylations per nucleotide follow a more complex function but they follow it from a very high dose down to 5 mg/kg. I don't really understand the kinetics of that, but the point is that there is no break in the effect; it gets in at high doses and low doses, the function is smoothly continuous.

Dr. C. Kensler (*Arthur D. Little, Inc.*): You said the basic guideline when you extrapolate downward was linearly, unless one has evidence that that isn't the case. What kind of evidence would you accept?

Dr. Freese: Statistically significant experimental evidence showing the absence of a mutagenic effect at low doses.

Dr. Kensler: You mean a 10,000 mouse experiment.

Dr. Freese: Yes, that's exactly what I want to say. Unless one has experimental evidence at low doses, one has to extrapolate from the data obtained at high doses. To do that one certainly should not accept a threshold dose. There is a basic difference between a mutagenic effect and a toxic effect. A mutagenic effect alters single cells which then multiply; in a toxic effect a large number of cells must be affected, by inhibition or cell killing before significant toxicity is observed.

Dr. Kensler: One of the primary missions of the NCTR was to set up a megamouse experiment to try to tell the shape of the dose curve. Most people said this would be a waste of time and money.

Dr. Freese: I'm not saying that one should do it, but because it can't be done one has to extrapolate. One should not extrapolate to some assumed no-effect value, but one should extrapolate linearly down to zero dose.

Dr. Kensler: In our current status of ignorance I'd like to see us investigate the lower end of the dose curve, particularly for different kinds of chemical mutagens.

Dr. Cummings: One more point with regard to the Generoso data. I think that the data do not say that there is a threshold. The data with regard to dominant lethals simply say that you reach a point at which it can no longer be measured. That's a different thing; in fact, there is a positive effect with regard to translocation at the lowest dose measured. It would probably be possible to measure a translocation frequency at even lower doses by doing a larger experiment. The data really agree with your interpretation.

Dr. Freese: Some of these issues are semantic. The question is how you want to extrapolate the data. You may decide that you want to extrapolate the experimental curve down to that dose which will give you a no-effect point or you may decide to take the lowest experimental value and extrapolate it linearly down to zero concentration, which will give some effect at any dose.

Dr. Cummings: Right. I don't know of any data that would suggest that there is a real threshold.

Dr. J. F. Crow (*Univ. of Wisconsin*): I wanted to reiterate Dr. Freese's point. When we say linear extrapolation we don't really mean to fit a least-squares line to the existing points and carry that back as far as it goes. What we really mean is to connect the existing points with the zero effect and regard that as linear. Maybe we need a better vocabulary.

Dr. B. Bridges (*Univ. of Sussex*): I think that I would very much agree with what you say, that you cannot assume that for genetic effects in a cell there is a no-effect threshold. On the other hand, I can see the toxicologist point of view—that the substance may not reach the target organ. It seems to me that we have a real problem here, in that many environmental mutagens, two that I've worked with, both mopped up pretty strongly in the body. The concentration gradient from the route of entry to the gonads can be very steep—so steep that I doubt whether the pharmacologists can measure it—and this makes it exceedingly difficult to do any

calculations. The toxicologists may believe firmly that there is no mutagen in the gonads, and they may be right. Certainly it will be quite clear that there is less than a linear extrapolation back. It seems to me this is really where we are going to come across some major disagreements in the future.

Dr. Freese: In that connection I would say that we are not only concerned about mutations in gonads, impairing future generations, but also about somatic mutations affecting the present generation. If a compound is mutagenic we still don't want it in the population because we don't want a carcinogenic effect.

Dr. L. Friedman (*FDA*): As probably the only card-carrying toxicologist present, when we start talking about thresholds and no thresholds I think we really are, in the present state of things, getting into an area where we are not going to convince each other by argumentation. I would like to make the point that in considering a phenomenon such as mutation in a very general respect, where, as you point out, you are starting with a background level, you automatically have no possibility of demonstrating a threshold because you already are beyond a threshold, so the argument falls right there. The issue, it seems to me, is not the general one as to whether you see the threshold in a general sense but whether any particular substance you study which may or may not be out there in the environment is contributing enough in itself to make a significant addition to background. When you begin to think about this kind of possibility, you must start from a basic postulate and make some logical inferences based on general knowledge. I think we all agree the capability of doing experiments at such low levels does not exist at the present time. We can't even think of measuring the doubling rate; we're not about to measure 1/100 of 1% increase or something like that which would satisfy us about a threshold phenomenon. But when you start to think in these small numbers, the argument inevitably gets back to the smallest number to the one molecule. Your argument inevitably gets into that kind of a framework; then, to me at any rate, it becomes an intellectually repugnant concept that you have no threshold, if you consider that a cell has something like 10^{12}–10^{14} molecules and membranes, structures and barriers of all kinds. The probability of a molecule reaching whatever the target is becomes infinitely small even given an infinite time, so to talk glibly about whether or not there is a threshold I think serves no purpose. In the practical sense, I think, for mutation we've already exceeded the threshold; the issue in any particular case is what is the evidence that we are actually increasing the hazard. That's not such a simple matter, I know. We tend to oversimplify and make things appear very easy by drawing these straight lines wherever we decide we should draw them. This is another question—where do we decide to draw them from.

Dr. Freese: I think what we are trying to do is to put the burden of proof upon those who argue that there may be a threshold. To argue for a threshold with respect to a very small number of reagent molecules is as meaningless as to state that a chemical reaction requires a certain minimal number of molecules if it is to take place. Whether we can measure it or not, the chemical reaction still occurs, and the reaction of individual molecules is governed by statistical mechanisms.

Dr. S. S. Epstein (*Case Western Reserve*): As another card-carrying toxicologist I'd like to make a couple of points. First of all I think it would be helpful in situations like this to talk about apparent no-effect levels rather than no-effect levels. Toxicologists are very fond of calling these no-effect levels, based on experiments of 30–40 mice or rats and from such concepts of no-effect levels they proceed to equate no-effect levels with threshold. This clearly is statistically nonsensical; it would be much simpler if one talked about these as apparent no-effect levels to make very clear the artifactual situation, namely: a very small experiment of 30 or 40 animals compared to millions or hundreds of millions of people at risk.

The second point I wanted to make, the mere fact that you have a natural background of environmental mutagens or carcinogens is not in any way relevant to the concept of threshold. The fact that we have a background of environmental mutagen I presume has some relationship to mutagenesis in man and the adverse mutations and the high incidence of cancers that man has suffered since time immorial. To suggest that the existence of a natural background of environmental carcinogens and mutagens therefore brings the concept of threshold in the relationship to the introduction of new synthetic agents into the environment is a non-sequitur.

Dr. S. Abrahamson (*Univ. of Wisconsin*): I think Dr. Friedman was referring to a background mutation rate and not a background of environmental mutagens, but perhaps Dr. Friedman would like to rebut.

Dr. Friedman: I'd like to clarify the issue a little. Toxicologists do not talk about no-effect levels. When they use the terminology it is always in the context of experimental no-effect levels. We don't talk about apparent levels; we talk about the results of a given experiment. In that case, the experiment has shown a no-effect level with a certain degree of probability, and that's what we talk about.

Dr. Epstein: May I just make one point. This concept of no-effect level is equated in toxicological circles to threshold levels. When you see a no-effect level in an experimental situation, that is proper in relation to that experimental situation. If you want

to extrapolate from that, talk about it as an apparent no-effect level but don't get mixed up with thresholds.

Dr. Abrahamson: I really think that Dr. Bridges answered this question yesterday when he asked what the effect is at those concentrations to which human beings are exposed and whether we can tolerate it if the agent is a valuable one.