Chapter 1

Dose and Time Determining, and Other Factors Influencing, Toxicity

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1.1 INTRODUCTION

This chapter is intended as an introduction to the toxicity of pesticides and an evaluation of methods for their study. This chapter is not meant as a set of guides for testing a particular compound intended for a particular purpose. Of course, the importance of such guides is recognized. In addition to offering suggestions about where details on technique may be found, an effort is made to identify (a) parameters in need of special study and (b) the variety and limitations of present approaches to such study. Under the circumstances, it has seemed best to organize the discussion of techniques conceptually and not in the usual operational way according to acute and chronic tests, dermal toxicity, and the like. Briefly, the statistical and other methods for studying toxic reactions in intact animals are discussed in this chapter. Methods for studying absorption, distribution, metabolism, storage loss, and excretion are considered in chapters on "Pesticides Disposition" as well as techniques for measuring different kinds of injury and injury in different tissues. Methods measuring exposure and quantitative metabolism in people under various other practical conditions are also discussed there.

We (Rozman and Doull) considered this chapter by Dr. Hayes to be the best summary of thus far recognized fundamental principles and unresolved problems of toxicology long before we were asked to revise it. We recognized that any attempt to revise a scholarly activity of such high quality is not without risk of ending up with an inferior product. Therefore, the decision was reached together with the Editor in Chief that the chapter as written by Hayes (1991) will be retained in its original form, but that we would add a discussion after each section and subsection when such an addition is warranted, to bring in the more recent developments regarding the role of time and kinetics in toxicology. As Hayes points out in this chapter, toxicology needs a unifying theory. In the following sections, we outline our proposal for such a theory and indicate at the proper passages how it would amplify Hayes' writings.

1.1.1 Dose and Time as Fundamental Variables of Toxicity

Toxicity *(T)* is a function of exposure *(E)*, and *E* is a function of dose *(c)* and time *(t)* $[T = f(E(c, t)]$. Toxicity is the manifestation of an interaction between molecules constituting some form of life and molecules of exogenous chemicals or physical insults. Consequences of molecular interactions or physical insults may propagate, through causality chains, all the way to the organismic level (Rozman and Doull, 2000; Rozman *et al*., 2006). There are two fundamental ways to view this interaction: (1) What does an organism do to a chemical? (2) What does a chemical do to an organism? Dealing with the first question led to the development of the discipline of pharmacokinetics, which was later incorporated into some toxicity studies and therefore, in that context, it would be more appropriately called toxicokinetics *(K).* The other question was addressed by the discipline of pharmacology in the form of pharmacodynamic experiments, which again in the context of toxicity would be more properly termed toxicodynamics *(D).* We recognize that the use of the prefixes pharmaco- and toxico- in the context of kinetics and dynamics is problematic because both involve value judgements not compatible with the unbiased interpretation of (a) law(s) of nature. However, before this issue can be sorted out in terms of epistemology, we will be using these terms interchangably and often in the traditional (perhaps incorrect) way. Thus, toxicity *(T)* may be defined as a function of *E, K,* and *D.*

$$T = f(E, K, D)$$

This functional relationship can be described mathematically by a simple differential equation using the chain expansion:

$$\frac{dT}{dE} = \frac{dT}{dD} \cdot \frac{dD}{dK} \cdot \frac{dK}{dE}$$

A definition of toxicity according to Rozman and Doull (1998) runs as follows: "[toxicity] is the accumulation of injury over short or long periods of time, which renders an organism incapable of functioning within the limits of adaptation or other forms of recovery." This definition implies that toxicity is a function of time in addition to the dose. This concept was already recognized by Paracelsus 500 years ago. A closer scrutiny of the definition of toxicity indicates that the relationship between toxicity, dose *(c),* and time *(t)* is a complex one because toxicokinetics itself is dose- and time-dependent *[K = f(c, t)]* as is toxicodynamics *[D = f(c, t)]*. It should be noted that the various time-dependencies seldom run on the same timescale.

Conceptually, *K* may also be viewed as a function of the dynamic change between absorption (Abs) and elimination (El),

$$K = f(\text{Abs}, \text{El})$$

because it is the ratio between entry rate (absorption) and exit rate (elimination) that determines the time course of a compound in an organism. In the simplest case of an intravenous bolus injection (instantaneous absorption), the time course is determined by the rate of elimination alone for a compound obeying a one-compartment model. Usually absorption is faster than elimination, making processes related to elimination (distribution, biotransformation, excretion) rate-determining or -limiting in most instances.

In analogy, *D* may be viewed as a function of the dynamic change between injury *(I)* and recovery *(R),*

$$D = f(I, R)$$

because it is the ratio of injury to recovery that determines the time course of an adverse effect in an organism. The simplest case for such an injury would be when an organism would recover from an acute injury in accordance with a one-compartment toxicodynamic model. Again, processes related to recovery are usually slower than the rate of injury. Therefore, more often recovery (adaptation, repair, reversibility) will be rate-determining or -limiting.

Most often compounds do not behave in an organism according to a one-compartment model. The reason for this is that elimination from the systemic circulation itself can be a function of excretion (Ex), distribution (Dist) and biotransformation (Bio).

$$\text{El} = f(\text{Ex}, \text{Dist}, \text{Bio})$$

When any or all of these processes become rate-limiting, two or multi-compartmental models are needed.

Again, in analogy to *K,* recovery *(R)* in a *D* model may not be a simple function of, for example, reversibility (Rv), but could also require repair (Rp). In addition, adaptation (Adp) may also be occurring:

$$R = f(\text{Rv}, \text{Rp}, \text{Adp})$$

In such instances, two- or multi-compartment D analyses are needed to describe the toxicity of a compound that affects any or all of these processes. Absorption and injury can be thought of as being analogous manifestations of *K* and *D.* Absorption is a function of site (S) and mechanism *(M),* as is injury:

$$\text{Abs} = f(S, M)$$

$$I = f(S, M)$$

This analysis can be continued all the way to the molecular level. It is clear that any rate-determining step or rate-limiting steps, originating at the level of molecular interactions, will then propagate through causality chain(s) to the levels depicted in Fig. 1.1, which represents a schematic illustration of this concept.

Each of the processes depicted in Fig. 1.1 may be dose- and time-dependent although past experiments often failed to demonstrate this, because they were conducted with preponderant emphasis on one or the other; for example, *D* was mainly studied as a function of dose and *K* mostly as a function of time.

Time has always been an important factor in designing toxicological experiments, yet time as an explicit variable of toxicity has been afforded very little attention. It is interesting that, after Warren (1900) was severely criticized by Ostwald and Dernoscheck (1910) for his analogy of $c \times t = k$ to $P \times V = k$ of ideal gases, the entire issue was forgotten. Even though $c \times t = k$ kept surfacing repeatedly (e.g., Druckrey and Küpfmüller, 1948; Flury and Wirth, 1934; Littlefield *et al*., 1980; Peto *et al*., 1991) an analogy to thermodynamics was not contemplated again, at least not to our knowledge! When we "rediscovered" the $c \times t = k$ concept in still another context (delayed acute oral toxicity) this required some reevaluation of the role of time in toxicology in both a historical context and as an independent variable.

Ostwald and Dernoscheck (1910)'s analogy of toxicity to an adsorption isotherm is problematic, because adsorption entails processes that are far from ideal conditions. Much more reasonable is Warren (1900)'s analogy to $P \times V = k$ for ideal gases as a comparison for ideal conditions in toxicology. Reducing the volume of a chamber containing a given number of molecules or atoms of an ideal gas will decrease the time for any given molecule or atom to collide with the wall of the chamber. This leads to increased pressure, which is simply an attribute of the increased number

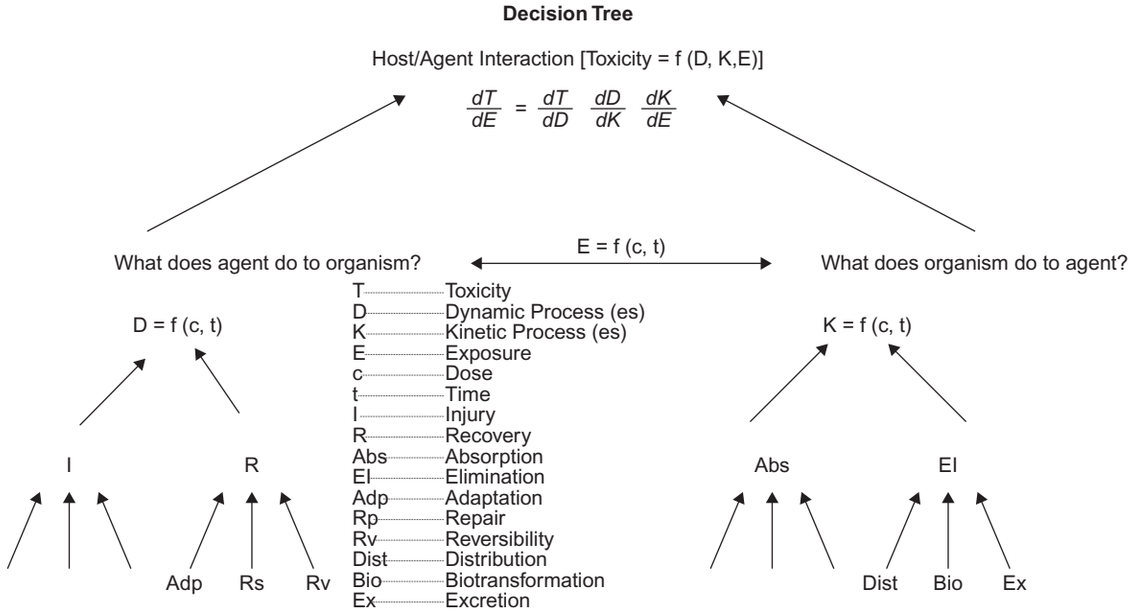

FIGURE 1.1 Conceptual outline of the decision tree approach.

of molecules per unit volume, which is concentration. Thus $c \times t = k$ and $P \times V = k$ are compatible with each other if looked at mechanistically. Of course, Ostwald and Dernoscheck's comparison of toxicity to an adsorption isotherm is much closer to the real-life situation of toxicology, where the most frequent finding is that ($c \times t^x = k$).

These thought experiments and some discussions led to the recognition that toxicologists did everything the opposite way of thermodynamicists. Instead of starting out with the simplest model (ideal gas in thermodynamics corresponds to ideal conditions in toxicology experiments) and building into it step by step the increasing complexity of the real world, toxicologists tried to predict from one complex situation to another complex situation. In addition, time as an explicit variable was largely ignored although it is one of two fundamental variables of toxicity (Rozman, 1998). It is unlikely that a better understanding of biological processes at the molecular level alone will lead to improved risk predictions in toxicology, as long as the experimental designs of toxicological studies provide the wrong reference points for departure from ideal to real conditions. For example, the standard inhalation toxicity protocols (6 h/day, 5 days/week) cannot yield $c \times t = k$ because after 6 h of intoxication, there are up to 18 h of recovery, and on weekends there are up to 66 h of recovery, at least for compounds of short half-life. This would require at least two additional functions to correct for departure from kinetic steady state. The real-life situation is even more complex where departures from the ideal condition (steady state) are highly irregular. Nevertheless, it is reasonable to expect that risk predictions will be possible for even the most irregular exposure scenarios once the reference points are established as dose-and time-responses under ideal conditions (toxicodynamic or toxicokinetic).

In 25 years of studying the toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and related compounds, the concept of $c \times t = k$ did not emerge in any other experimental context except in the two recent subchronic-chronic studies, which were conducted under conditions of toxicokinetic steady state (Rozman *et al*., 1996; Viluksela *et al*., 1997, 1998). Nevertheless, a general interest in the role of time in toxicology pervaded the herein-presented line of thinking for many years (Rozman *et al*., 1993; Rozman *et al*., 1996; Rozman and Doull, 1998; Rozman, 1998). Most toxicologists are familiar with Haber's rule of inhalation toxicology and its applicability to war gases and some solvents. Much less attention has been given to Druckrey's work, which extended the $c \times t$ concept to lifetime cancer studies by oral rather than inhalation exposure. Finally, there is very little cross-referencing of the $c \times t = k$ data that were generated by entomologists (e.g., Peters and Ganter, 1935; Busvine, 1938; Bliss, 1940) and those established by toxicologists. History demonstrates that a fundamental relationship in science keeps reappearing in different contexts as is the case with $c \times t = k$. During this period many apparent exceptions seem to be occurring with no satisfactory explanation. Attempts at generalization usually fail until a commonality is detected among all experiments as in this case among those that yielded $c \times t = k$. This commonality is toxicokinetic steady state and/or irreversibility of an effect, which of course can be interrelated. Anesthesia, like intravenous infusion, leads to rapid and sustained steady state for compounds of short half-life. Most anesthetics and solvents do have short half-lives and many obey Haber's rule, except when

measurements are taken while an adaptive process is underway, that is, induction of a protein. Druckrey and the ED 01 study used feeding as a route of exposure, which yields a better steady state for compounds of intermediate half-life than, for example, gavage. However, the exponent x in the term of Druckrey's general formula increases above 1 rapidly as the half-life of compounds becomes shorter, because there is intermittent recovery between bouts of feeding. Most of the entomology studies were related to fumigation, which often but not always resulted in fairly rapid steady state. Finally 1,2,3,4,6,7,8-heptachlorodibenzo-*p*-dioxin (HpCDD), which has a half-life of 314 days (Viluksela *et al*., 1997) in female rats, yields virtual steady state for a 70-day observation period after any route of administration but TCDD, with a half-life of 20 days, does not. However, when TCDD's toxicity was studied under steady state conditions, its subchronic-chronic toxicity also occurred according to $c \times t = k$ (Rozman *et al*., 1993; Saghir *et al*., 2005).

1.1.2 Definition of Dose and Time

Before analyzing dose-time relationships further, it is useful to establish clear definitions of these fundamental variables of toxicity. Historically, neither dose nor time has been defined with clarity as a variable of both toxicokinetics and toxicodynamics. It is customary to use the terms acute dose and acute effect as if the two were interchangeable. In fact, an acute dose can lead to chronic effects (Druckrey *et al*., 1964) and multiple doses can trigger a fulminant episode (Garrettson, 1983) of toxicity. In risk (safety) assessment it is always the total dose delivered that is of concern, although in therapeutics the daily dose is often referred to simply as the dose. Therefore, a useful definition of dose in toxicology, would be:

$$\text{Dose(c)} = \sum_{n=1}^{n} \textit{dose rates}.$$

According to this definition a single acute dose would represent the limiting case when the dose rate equals the dose. This definition would be valid for any kind of irregularity in the dosing regimens and is analogous to the definition of dose in radiation biology.

Ever since the dawn of human consciousness, mankind has struggled with the notion of time. It is not possible to predict what influence the concept of toxicological time will have on our perception of time. Suffice it to say at this junction, it is not possible to think of toxicity without the implicit presence of time as a variable, although in toxicity studies, time received only semiquantitative designations (acute, subacute, subchronic, chronic). In fact, one could view organisms as instruments exquisitely sensitive to time. Important for toxicology is the concept that the time course of a toxicant in an organism (kinetics) is very often different than the time course of toxicity (dynamics). Underlying biological processes (absorption, distribution, elimination, injury, adaptation, recovery) have their own timescales depending on the molecular events behind each process (e.g., enzyme induction, receptor regulation either directly or via gene expression). Thus, in toxicology the dose is a pure variable, but there are many different processes occurring on different timescales yielding different $\int c\ dt$ integrals leading to complex interactions, which can be described as $c \times t^x$. In spite of this complexity, science can deal with it in a traditional, analytical fashion. Because only knowledge of rate-limiting steps is required to accurately describe toxicity, this will often reduce complexity to manageable proportions.

1.1.3 Dose and Time Relationships

Toxicity is a function of exposure and exposure is a function of dose and time $[T = f[E(c,\ t)]]$. Consequences of interactions between a toxic agent and an organism at the molecular level propagate through toxicodynamic or toxicokinetic–toxicodynamic causality chains all the way to the manifestation of toxicity at the organismic level (Fig. 1.1). If the recovery (consisting of adaptation, repair, and reversibility) half-life of an organism is longer than the half-life of the causative agent in the organism then toxicodynamics becomes rate-determining (one-compartment model) or rate-limiting (multicompartment models) (Rozman and Doull, 2001a). If the toxicokinetic half-life of the compound is longer than the recovery half-life, then toxicokinetics will be rate-determining (-limiting), in which case the toxicokinetic area under the curve (AUC) will be identical to the toxicodynamic AUC. There are three limiting conditions for $c \times t = k$ to emerge when the causality chain propagates through either toxicodynamic or toxicokinetic-toxicodynamic processes:

Toxicodynamics

1. In case of no recovery (no reversibility, no repair, no adaptation) linear accumulation of injury will occur according to a triangular geometry $(c \times t/2 = k)$ following repeated doses or according to a rectangular geometry after a single dose $(c \times t = k)$, provided that the $c \times t$ lifetime threshold has been exceeded, which occurs when $c_{threshold} \times t_{lifespan} = k$.
2. After recovery (reversibility, repair, adaptation) steady state has been reached, injury will occur according to a rectangular geometry $(c \times t = k)$, after exceeding the $c \times t$ lifetime threshold.

Toxicokinetics

1. No elimination will lead to linear accumulation of a compound and as a consequence to accumulation of injury according to a triangular geometry $(c \times t/2 = k)$ after repeated doses or according to rectangular geometry after a single dose $(c \times t = k)$ above the $c \times t$ lifetime threshold.

2. After toxicokinetic (and as a consequence toxicodynamic) steady state has been reached, injury will occur above the $c \times t$ lifetime threshold according to a rectangular geometry *($c \times t = k$)*.

Exposure Frequency

As the toxicokinetic and toxicodynamic half-lives become shorter and shorter the distinction between elimination and recovery half-lives becomes less important, because another time dependence, that of the frequency of exposure, starts dominating the time dependence:

1. Compounds having very short toxicokinetic or toxicodynamic half-lives will reach steady state rapidly and yield $c \times t = k$ upon continuous exposure according to a rectangular geometry above the $c \times t$ lifetime threshold provided that adaptation and repair are also at steady state.
2. Other types of geometries certainly can be created by elaborate, but regular dosing regimens. These scenarios are less likely to play a practical role in toxicology, although they may be of theoretical interest in the establishment of model parameters for predicting toxicity after irregular dosing regimens.

It should be kept in mind that the mathematics of first-order processes, when appropriate, are valid for bimolecular reactions (e.g., receptor binding), which result in the propagation of the causality chain to the level of modeling (Fig. 1.1). Therefore, 90% of toxicodynamic steady state will not be reached until 3.32 recovery half-lives have elapsed. Thus, Haber's rule will be obeyed only if the observation period is outside of about 4 recovery half-lives or if recovery is a zero order process.

Thus, the various *($c \times t = k$)* scenarios represent limiting conditions (Rozman and Doull, 2001b). The magnitude of the $c \times t$ product is a function of the potency of the compound, of the susceptibility of the organism, and of the deviation from the ideal conditions and will yield $c \times t^x = k$ for nonlimiting conditions. It should be recognized that the dose *(c)* does not have inherent exponential properties, but time *(t)* does have such properties, because under nonideal conditions toxicity is a function of at least two independent timescales: one being the half-life of the rate-determining step (toxicodynamic and/or toxicokinetic-toxicodynamic) of the intoxication (intrinsic property of organism or compound), the other one being the frequency (which includes duration) of exposure, which is independent of both the compound and the organism.

In conclusion, these data and consideration of a significant body of evidence accumulated over the last 100 years suggest that $c \times t = k$ is part of a fundamental law of toxicology, and possibly of biology in general, that can be seen only under ideal conditions (Rozman and Doull, 2001b). It must be emphasized that the dimension of the $c \times t$ product is not energy but effect (Rozman, 2008) as in action (Wirkung). This has been confirmed using other classes of compounds and the herein-described ideal conditions (Saghir *et al*., 2005). Therefore, Paracelsus' famous statement should be supplemented to read "Dosis et tempus fiunt (faciunt) venenum" (Dose and time together make the poison). Implications for risk assessment are that the margin of exposure (MOE) must be defined in terms of both dose and time. This can be done by relating the real-life (discontinuous) exposure scenario to that of ideal (continuous) exposure condition:

$$\text{MOE} = \frac{c \times t^x}{c \times t}$$

The margin of safety and its reciprocal, the margin of risk, can be determined when the MOE exceeds the $c \times t$ lifetime threshold (Rozman and Doull, 1999, 2000).

Figure 1.1 may also be used as a decision tree to identify critical steps needed for modeling to predict toxicity. It is important to note that both a high degree of irreversibility and toxicodynamic steady state are rare phenomena in toxicology, although both can be seen any time the observation period is much shorter than the recovery half-life. In real-life situations there are usually at least two or three rate-limiting steps in toxicokinetics and likely as many in toxicodynamics. It must be emphasized, though, that multiple toxicokinetic compartmental models do not necessarily require multiple toxicodynamic models, and vice versa. However, if there are three different rate-limiting processes occurring on different timescales in toxicokinetics and three different rate-limiting processes taking place on three different timescales in toxicodynamics, such a scenario would represent a formidable computational task for a theoretical treatise on risk assessment. Therefore, a practical approach would be to conduct experiments at toxicodynamic steady state (which of course would require a preexisting toxicokinetic steady state in many instances) as a point of reference clearly defined by $c \times t = k$. Then, experiments would need to be carried out for different compounds with different half-lives to establish model parameters, which describe departures from toxicokinetic-toxicodynamic steady state of increasing frequency and irregularity.

In summary, $c \times t = k$ represents the most efficient (a kind of worst case) exposure scenario for producing an effect, namely, continuous exposure till manifestation of an effect. Experimentally, this condition is often met by continuous inhalation exposure or daily oral administration of compounds that have toxicodynamic-toxicokinetic half-lives of a few days or longer and /or effects that are largely irreversible. It must be emphasized that any departure from the worst case scenario will result in a change of $c \times t = k$ into $c \times t^x = k$. Departures are represented by regular or irregular interruptions of exposure and/or intermittent recovery from injury. The larger the departure, the larger will be x, indicating that increasing x is equivalent to

decreasing toxicity. This is entirely logical, when recognizing that increasing interruptions of exposure and/or injury will result in longer and longer periods of time needed to cause toxicity equivalent to that of continuous exposure, because of increasing intermittent recovery. To express this more clearly, we can write

$$c \times t^x = k \text{ or } c \times t \times t^{x-1} = k$$

Thus, t^{x-1} is a simple transforming factor which changes the slope of the log c vs log t plot back to unity. It may be viewed as the toxicological timescale of recovery which runs counter to the timescale of toxicity, thereby reducing it.

A limiting condition for first-order processes will be reached when exposure occurs outside of 6.64 toxicokinetic-toxicodynamic half-lives, because at that time 99% elimination/recovery will have occurred. Under such conditions (which are closest to the real-life situation for most compounds), toxicity will be less dose- and time-dependent. In this case mainly the frequency of exposure will determine x. If x, is then determined experimentally, for say 1, 2, 4, 8, 16, and 32 days for a compound with a toxicokinetic or toxicodynamic half-life <3.6 h after continuous vs intermittent exposure under isoeffective conditions, then plotting of the data will allow extrapolation to any exposure scenario outside of 6.64 half-lives (which corresponds to 1 day). Most dietary constituents fall in this category. For zero-order processes two half-lives are needed for elimination and/or recovery. It should be kept in mind that the half-life of zero order processes (unlike that of first-order processes) is concentration-dependent.

A series of articles has explored how other disciplines deal with complex systems (Goldenfeld and Kadanoff, 1999; Koch and Laurent, 1999; Weng *et al*., 1999; Whitesiles and Ismagilov, 1999). Goldenfeld and Kadanoff (1999) made some important observations which are relevant for toxicology. Simple laws of physics give rise to enormous complexity when the number of actors is very large. We have the same paradox in toxicology in that the $c \times t$ concept is very simple, but the "real-world" manifestation of toxicity is very complicated. One other observation is equally relevant: "Use the right level of description to catch the phenomena of interest. Don't model bulldozers with quarks." This translates in toxicology to: Don't model toxicity at the molecular level. The decision tree approach in Section 1.1.1 (Fig. 1.1) was developed to aid toxicologists and modelers to identify both the appropriate phenomena and the right level of modeling. Toxicologists can avoid much unnecessary experimentation by using this top-to-bottom approach rather than the currently fashionable bottom-to-top approach.

1.1.4 Analogy to Thermodynamics

In physics, Boyle's law of ideal gases gave rise to thermodynamics, and molecular and mechanistic considerations led to a theory of gas reactions. The former is based on the idea of finding the minimum number of fundamental variables that can describe the simplest possible dynamic system ($P \times V = k$ for ideal gases). The latter required a great deal of knowledge about the mechanism of chemical reactions (wall reaction, activation energy, etc.). Both of these approaches have been attempted in toxicology with, as yet, limited success, as we shall see in subsequent discussions. The reason for the lack of advance in theoretical toxicology is probably that, unlike thermodynamicists, we did not start out by defining the simplest possible toxicological conditions with a minimum number of variables as a point of departure toward more complexity, although coincidentally experiments were conducted under such ideal conditions and in every such instance Haber's rule proved to be applicable (e.g., Gardner *et al*., 1977), even though authors may have failed to notice it (Sivan *et al*., 1984).

The lack of conceptualization of the three variables of toxicity resulted in arbitrary study designs, which further eroded the predictability from one experiment to another. It is our opinion that analogous considerations to thermodynamics might help to optimize study design and eventually to build a theory of toxicology. Thermodynamics like toxicology has three fundamental variables (*P, V,* and *T* vs *c, t,* and *W*). *W* (*Wirkung* in German) will be used for effect, because of the many Es (exposure, elimination, effect, excretion) in English. Before the development of a comprehensive theory of thermodynamics, it was clear to scientists that, to study an independent and a dependent variable, a third or other variables had to be kept constant. We have not done this in toxicology, although most dose-response studies were conducted at constant time (isotemporal). However, to study the relationship between time and effect, the dose needs to be kept constant (isodosic). Moreover, to examine the relationship between dose and time, the effect must be kept constant (isoeffective). The $c \times t$ product will not emerge from the equation of ergodynamics (Wirkungslehre) until after elucidation of the relationship between specific effect at constant time and specific effect at constant dose. In other words, we must learn more about k before significant theoretical advance is possible (Fig. 1.2).

As mentioned before, most experiments were conducted isotemporally in the past (14 days, 90 days, 104 weeks), which is appropriate for dose-response studies. The arbitrary choice of these time points and the inexactitude of diagnosis (stuff them and count them) led to a great deal of confusion in the 14-day studies, because different dose responses, meaning different mechanisms, were often lumped together. Experiments in toxicology have frequently been conducted under isoeffective conditions, mostly with the end point being 100% of an effect (mortality, cancer). However, systematic investigation of $c \times t = k$ has not been done, for example, at 20 or 80% of an effect. Finally, there are very few experiments that were conducted under isodosic conditions, because this requires that the concentration be kept constant at the site

Ergodynamics (Wirkungslehre)

$$dW = \left(\frac{\partial W}{\partial c}\right)_t dc + \left(\frac{\partial W}{\partial t}\right)_c dt$$

dW = 0 isoeffective

$$-\left(\frac{\partial W}{\partial c}\right)_t dc = \left(\frac{\partial W}{\partial t}\right)_c dt$$

dt = 0 isotemporal

$$dW = \left(\frac{\partial W}{\partial c}\right)_t dc$$

dc = 0 isodosic

$$dW = \left(\frac{\partial W}{\partial t}\right)_c dt$$

FIGURE 1.2 Conceptualization of ergodynamics (Wirkungslehre) at constant action (Wirkung), at constant dose, and at constant time.

of action. The only experiment-driven condition, other than aquatic and *in vitro* toxicology, that keeps the concentration at the site of action constant is continuous inhalation exposure. For example, Gardner *et al*. (1977) have reported such data after continuous inhalation exposure of experimental animals to benzene and SO_2 when the end point in question was measured immediately after termination of exposure (chronaxy, leukopenia). However, when the end point of measurement (streptococcal infection-related mortality) did not occur immediately after cessation of NO_2 exposure the time response started flattening out (Gardner *et al*., 1979). A systematic investigation of these issues has been done recently for HpCDD after oral administration with as yet only one end point of toxicity (delayed acute toxicity) as end point of measurement (Rozman, 1999), although confirmation of the analysis is emerging for anemia and lung cancer as well (Rozman, 2000b; Rozman *et al*., 2005). These data provide support for the suggestion of Rozman *et al*. (1996) that viewed the dose-time-response as a three-dimensional surface area similar to but conceptually distinctly different from the traditional model of Hartung (1987). Experiments conducted under isoeffective conditions (slices parallel to the dose-time plane) correspond to Haber's rule of $c \times t = k$ represented by hyperbolas. Studies carried out under isotemporal conditions (slices parallel to the time-effect plane) yield S-shaped dose-response curves along which $c \times t = k \times W$ whereas isodosic investigations (slices parallel to the dose-effect plane) produce S-shaped time-response curves along which $c \times t = k \times W$ also. Indeed plotting of the $c \times t$ product against W (effect) for HpCDD for doses causing about 10–90% wasting or hemorrhage yielded a straight line (Rozman, 2000a) of high correlation ($r^2 = 0.96$). This is a beginning core of a theory of toxicology, analogous to $P \times V = k$ for isotherms and $P \times V = k \times T$ for isobars or isochors. Of course thermodynamicists know that $k = n \times R$, but toxicology is

Ergodynamics

$c \times t = k \times$ Effect (Wirkung)

Thermodynamics

$$P \times V = n \times R \times \mathrm{T}$$

$$dW = \left(\frac{\partial W}{\partial c}\right)_t dc + \left(\frac{\partial W}{\partial t}\right)_c dt$$

***W*.....Effect [Action (Wirkung)]**
***c*.....dose**
***t*.....time**

Physics

Action (Wirkung) = Energy × Time

Toxicology

Effect (Wirkung) = Dose × Time

FIGURE 1.3 Analogy between ergodynamics and thermodynamics.

not yet there. What is already clear at this junction, however, is that the dimension of $P \times V$ is energy, whereas the dimension of $c \times t$ is mass (energy) × time = action, which is called effect in toxicology (Fig. 1.3).

The action of a chemical may also be defined as

$$A = A_{\mathrm{NS}} + A_{\mathrm{S}} \quad \text{where } A_{\mathrm{S}} = \int c\, dt$$

total action *(A)* consisting of nonspecific action (A_{NS}) plus specific action (A_S). The nonspecific action is comparable to heat in thermodynamics that dissipates without being converted to work by an expanding gas. A chemical may have several specific actions (toxicodynamic) such as enzyme induction porphyria and liver cancer. At the same time the organism may have specific or nonspecific actions on the chemical. That portion of a dose which is converted to anything else but the effect of interest must be viewed as nonspecific action with regard to this particular effect. For example, if a chemical is rapidly converted to a much less toxic metabolite and eliminated, then such a compound will have very little specific action. In the case of metabolic activation only the portion of the dose which is converted to the more toxic metabolite will constitute the specific action and the rest must be viewed as nonspecific action. For example a suprathreshold dose of TCDD will be very efficiently converted to specific toxic action as there is very little biotransformation taking place and the toxic moity is TCDD itself. Therefore, in this case kinetics drive the toxicity of TCDD. Dynamics (binding to DNA) is the driving force for the toxicity of nitrosamines and they

require metabolic activation, which is just one of several possible metabolic pathways. Therefore, nitrosamines are less efficiently converted into toxic action than is TCDD.

Substituting for $c = k/t$ or for $t = k/c$ and integrating between c_1 and c_2 or t_1 and t_2 yields for isoeffective conditions another logarithmic form of Haber's rule:

$$\ln \frac{c_2}{c_1} = \ln \frac{t_1}{t_2}$$

for which an analogy also exists in thermodynamics.

1.2 KINDS OF TOXICITY

Toxicity may be classified according to the nature or the duration of the injury involved.

Toxicology traditionally has been defined as the science of the study of qualitative and, more important, quantitative aspects of injurious effects of chemicals and physical agents in a subject or in a population of subjects. Paracelsus had already recognized nearly 500 years ago that there is no such thing as nonpoisonous and that the dose alone makes a poison not to be poisonous. Even endogenous body constituents and foodstuffs can be deleterious to an organism if present in excessive quantities over prolonged periods of time. Thus, in addition to the dose, time is the second important variable with which the science of toxicology deals.

What then is toxicity? It is the accumulation of injury over short or long periods of time that renders an organism incapable of functioning within the limits of adaptation or other forms of recovery. Therefore, a more appropriate definition of the scope of toxicology would be that it is the science that elucidates the causality chain of interactions and their time course (exposure) between biological entities (subjects) of different intrinsic susceptibility and chemical and physical entities (agents) of different intrinsic potency. Thus, modern toxicology determines in a broader sense exposure responses consisting of dose-responses and time-responses thereby establishing practical thresholds which define the safety of chemicals.

1.2.1 Nature of the Injury

The kinds of injury or change that may be produced by chemicals and are known to be of practical importance in certain circumstances are acute and chronic toxicity in the restricted sense, neurotoxicity, teratogenesis, carcinogenesis, hypersensitivity, metabolism and storage, and induction of enzymes. The dosage-response relationships in these different kinds of toxicity or change are described in Section 1.4. Observed injury may be a direct result of the action of a toxicant or its metabolite(s), or it may be secondary to malnutrition, hormonal alteration, or some other change caused by the compound(s).

Hayes did not make a distinction between chemicals whose action is dominated by toxicodynamic processes and those whose action is determined by toxicokinetic processes. The decision tree in Section 1.1.1 (Fig. 1.1) requires identification of the rate-determining (or limiting) step(s). Accordingly, neurotoxicity, teratogenicity, carcinogenicity, hypersensitivity, and induction of enzymes are examples of toxico(pharmaco)dynamic processes, whereas metabolism (absorption, distribution, biotransformation, excretion) and storage are examples of toxico(pharmaco)kinetic processes.

1.2.2 Duration of the Injury

1.2.2.1 Factors in the Chronicity of the Injury

At least three major independent factors-compound, dosage, and duration of dosing-and a separately measurable dependent factor-storage-are involved in what is often lumped with misleading simplicity under the term "chronic toxicity."

Some compounds are inherently likely to produce chronic effects, which is largely the same as saying that their effects are highly irreversible. In some instances, a single dose not sufficient to produce any immediate effect or perhaps no detectable immediate effect, eventually leads to chronic illness.

It is important to realize that there is no necessary relationship between the number of doses and the chronicity of illness. If a material capable of producing chronic effects is administered repeatedly, the chance that chronic effects will occur is increased, and the chance that only acute poisoning will occur is decreased. However, both acute and chronic effects can occur as part of a single illness. Among the materials that can produce chronic illness by a single dose are thallium and arsenic (Moeschlin, 1965), triorthocresyl phosphate (Smith *et al*., 1930), or certain carcinogens (Bryan and Shimkin, 1943; Carnaghan, 1967; Magee and Barnes, 1962; Schoental and Magee, 1957). Undoubtedly some other materials such as lead often would cause both acute and chronic effects if absorbed at a sufficiently large single dose.

Other compounds such as potassium cyanide have produced only acute illness to this date. In other words, the illness caused by cyanide is similar whether it follows a single large dose or many somewhat smaller doses. If recovery occurs, it progresses at a rate determined by the severity of illness rather than by the number of doses received. The production of persistent effects is not characteristic of cyanide, although such effects may follow tissue anoxia of any cause.

Some compounds are intermediate to the examples cited in regard to the chronicity of their effects. Chronic poisoning cannot be produced by one drink of alcohol, but persistent excessive drinking can lead to chronic organic damage. There is considerable evidence that prolonged excessive intake of sodium in the form of table salt produces chronic hypertension (Meneely, 1966). In these instances, the easy

reversibility of the injury finally is overcome by prolonged high dosage.

Much confusion would be avoided if the expression "chronic poisoning" were restricted to chronic disease produced by a chemical or by the chemical changes secondary to radiation or other physical agents. Because chronic disease may be caused by a single dose and acute poisoning may follow repeated exposure, the duration of exposure ought to be specified separately.

Chronic illness (whether secondary to poisoning, infection, malnutrition, metabolic disorder, circulatory malfunction, neoplasia, genetic defect, or some unknown cause) is characterized not only by long duration but by certain pathological features, especially scarring and atrophy.

It is sometimes implied that chronic illness is necessarily obscure and difficult to diagnose. This simply is not true. Most of the poisoning produced by the alkyl mercury fungicides is both chronic and tragically obvious. Difficulty in diagnosis is more likely to be associated with very mild, transient illness or with failure to suspect the possibility of poisoning than with any particular set of clinical characteristics.

Hayes (1991) recognized the various relationships between manifestation of toxicity and dosing regimen. The decision tree approach in Section 1.1.1 (Fig. 1.1) provides a straightforward explanation for these various constellations. As the biological half-life of a compound increases (relative lack of elimination) or the recovery half-life from a toxic insult gets longer and longer (relative lack of recovery) the distinction between single dose rate and dose (total dosage) becomes blurred. In the limiting case of an infinite biological half-life (for a given life span) or an infinite recovery half-life (relative to life span) a single dose rate will cause the same degree of an effect as repeated dose rates of regular or irregular frequency. Departures from these limiting conditions will become more and more pronounced with decreasing elimination/recovery half-lives relative to the time to effect.

Hydrogen cyanide is a case in point for an effect in which recovery is the slower and hence rate-determining process. When the dose is high and the duration of exposure short, not much recovery can occur during time to death and $c \times t = k$ will be obeyed strictly. However, with decreasing dose and increasing time to death, recovery will play a greater and greater role in the overall outcome of toxicity with increasing departure from $c \times t = k$ (McNamara, 1976).

Alcohol represents the opposite end of the spectrum by causing highly reversible hepatotoxicity. Here it is not recovery from the toxic insult but accumulation of damage that is the slower and hence rate-determining process. Therefore, nearly lethal acute dose rates ingested repeatedly but with adequate recovery periods will not cause chronic hepatotoxicity (cirrhosis) because it is the accumulation of damage and not the injury itself that determines the time course of the disease.

1.2.2.2 Reversibility

The matter of reversibility is subject to several qualifications. Even when a chemical lesion is rapidly and completely reversible, as in the case of thiamin deficiency in its early stages, severe poisoning may lead to irreversible complications. Also, many compounds have two or more actions, which may differ in reversibility. Finally, the mode of action of many toxicants is unknown. It is, therefore, important to determine whether animals actually poisoned by a particular toxicant are capable of complete recovery or whether they are left with some residual functional or structural injury. As discussed in the preceding section, it is characteristic of some compounds to produce chronic illness (sometimes after a single dose) and of others to produce illness only if dosage is maintained at a sufficiently high level. Section 1.3.2 presents a quantitative method for recording the tendency of each chemical to produce cumulative *effects* following repeated dosing, and the related but separable phenomenon of cumulative *storage* of compounds or their metabolites is discussed later. The effects of many toxicants, including many pesticides, are fully reversible, but because a number of factors may be involved, the possibility of recovery must be tested directly for each compound. Unfortunately, little use is made of the technique of keeping the survivors of the higher doses of ordinary one-dose LD 50 tests for long periods without further dosing in order to observe possible latent effects. This technique is far from new. It has had some use in Great Britain in the systematic testing of pesticides. In fact, the procedure is recommended explicitly in the *Pesticides Safety Precautions Scheme Agreed between Government Departments and Industry,* issued by the Pesticides Branch of the Great Britain Ministry of Agriculture, Fisheries and Food (1966). The method is simple and capable of revealing the ultimate in irreversibility. It is especially suitable for discovering what Barnes (1968) has called *hit-and-run* poisons. As already mentioned a single dose of several natural and synthetic compounds have been shown capable of causing cancer and other chronic injury when administered orally or by other routes.

The possible reversibility of lesions produced by repeated doses is often neglected also. One can cite examples in which certain morphological changes of the liver have been called cancer without evidence of invasion or metastasis and without any effort to discover whether the changes would regress if dosing was to be discontinued. Such neglect represents not only poor toxicology but irresponsibility.

Reversibility is an important possibility, but is only one among several others whereby an organism can recover from injury. The other possibilities are adaptation and repair as discussed in Section 1.1.1 (Fig. 1.1). Storage of chemicals is related to their kinetics whereas hit-and-run poisons cause-injury by the dynamics of recovery. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), mirex, hexachlorobenzene (HCB), and polychlorinated biphenyls (PCBs)

are examples of chemicals that are stored in lipid and/or protein compartments of the body, which is the reason for their very long elimination half-lives. These very long half-lives dominate the manifestation of injury caused by these agents.

Opposite to these kinetically acting agents are the hit-and-run poisons such as warfarin and soman, both of which have very short elimination half-lives but long recovery half-lives; in the case of warfarin, in the form of reversibility (Nagashima *et al*., 1969); in the case of soman, in the form of repair (synthesis of new enzyme) (Rozman, 2000a).

1.3 QUANTITATION OF DOSAGE-RESPONSE RELATIONSHIPS

Scientific study of the effects of chemical or physical agents on living organisms requires measurement. A distinction is made between a measurement that involves an agent alone (dose) or one that involves an agent in relation to an organism (dosage). A 1.0 mg dose of a compound is identical, whether it is administered to a 20-g mouse or a 5000-kg elephant, but the dosages are vastly different: 50 mg/kg for the mouse and 0.002 mg/kg for the elephant. The susceptibility of different species or even different individuals can be compared precisely only if their body weight is also considered.

This does not mean that large animals always require a higher dose than small ones of the same species to manifest the same effect. The tendency in this direction may be obscured by individual variation, particularly because large animals frequently are older or better nourished than small ones and may differ in other ways also. Even so, the significance of individual differences of whatever origin can be studied most effectively if dosage rather than dose is considered.

The word dosage is properly applied to any rate or ratio involving a dose. Thus the expressions "milligram per kilogram" and "milligram per square centimeter" both designate a dosage. Dosages often involve the dimension of time [milligram per kilogram per day (mg/kg/day)] but the meaning is not restricted to this relationship.

The acute or one-dose ED 50, defined in Section 1.3.1.1 and generally expressed in terms of milligrams of material per kilogram of body weight, is the universally accepted primary way of expressing acute effects of solids and liquids that are swallowed, contaminate the skin, or are administered subcutaneously, intravenously, or by other parenteral routes. An LD 50 is a special case of an ED 50 in which the effect measured is death. The numerical form of these ED 50 or LD 50 values permits useful comparisons between the acute effects of different compounds or of the same compound administered by different routes. The 90-dose ED 50 (or LD 50) and the chronicity index both may help to express the results of repeated dosing. Finally the same mathematical procedure can be applied to studies of any duration, including lifetime studies.

The use of ED 50 and LD 50 values-whether for 1 dose or 90 doses-is the ideal way to express toxicity because these values are direct measures of the dosage received by fish or other organisms that live in water and obtain their oxygen from it. Under these circumstances, the investigator often must be satisfied with a statistical estimate of the time required for a given concentration to produce a specified effect in a fixed time.

No matter what the physical form of the chemical or the habits of the test species are, there is obvious interest in determining the largest dosage or concentration that produces no observable effect or no significant observable effect. This is the largest safe dosage for the test organism under the conditions of the test. Such a *no-effect-level* (NOEL) is often used as a basis for estimating a lower value considered safe under more varied conditions, including the exposure of other species, especially humans (Sections 1.2.7.4 and 1.5.9.1).

Finally, dosages may be compared in terms of tissue levels no matter what the physical form of the compound, the habits of the species, the route of absorption, or the duration of dosing.

The dosage-response relationship is the most fundamental single principle in toxicology. It extends to all kinds of injurious effects (Section 1.4) and implies the existence of a threshold dosage for each compound below which, under defined conditions, no harmful effect is produced (Section 1.3.7.4).

Hayes defined dose as mass (mg) and dosage as concentration (mg/kg). This is a useful distinction to generalize the "dosage-response" across species (e.g., mammals vs fish) after different routes of administration (e.g., oral vs dermal). It is also advantageous when applied to the concept of Hayes' index, which is an ingenious attempt to incorporate time as a variable of toxicity without designating time as an explicit function of toxicity. However, it is less accommodating for the herein-developed theory of toxicology, which uses both dose and time as explicit variables of toxicity. The most profound difference between the two approaches arises for safety and risk assessment. If toxicity is viewed as being solely a function of dosage as done by Hayes and others, then the logical consequence is to look for a no-observed-effect level (NOEL) or a lowest-observed-effect level (LOEL) as a point of departure for determining a safe dose. However, considering both dose and time as explicit functions of toxicity leads to having to incorporate both of these variables into safety and risk assessment as suggested by Rozman (2000a). The $c \times t$ concept provides a scientifically valid and firm departure point for safety and risk assessment instead of the inherent fuzziness of a NOEL or LOEL.

1.3.1 ED 50 or LD 50

1.3.1.1 One-Dose ED 50 or LD 50

An ED 50 is a statistical estimate of the dosage of a material that would produce a specified effect in 50% of a very large population of a test species under stated conditions, for example, a single oral dose of an aqueous solution given to male rats. Of course it would be impractical to use hundreds or even thousands of animals to make such a test. Even if this were done, it would be unlikely that the investigator would find the dosage to produce the effect in exactly half of the animals. That is why the parameter must be estimated statistically. In practice, test animals are divided into groups of moderate size, frequently about 10 per group. Each group is given one of a series of geometrically increasing dosages selected in such a way that the smallest dosage will produce the intended effect in only a small proportion of the group receiving it, whereas the largest dosage produces the same effect in the majority of animals receiving it. The result for each group is expressed as the percentage of animals showing the effect under study. By one technique or another, the percentage effect for each group is converted to a probit and related to the logarithm of the dosage that produced it. Any effect measured in this way must be recorded as an all-or-none response. However, phenomena that show continuous variation may be treated on an all-or-none basis merely by selecting an arbitrary limit. For example, systolic blood pressure may vary widely but could be made the basis of an ED 50 by counting all animals whose pressure exceeded 150 mm Hg.

Acute toxicity studies for purposes of approximating an LD 50 are conducted much less frequently today than in years past. However, acute lethality studies, if conducted properly, can provide a considerable amount of basic information about the toxicity of a chemical. Clearly, the amount of information obtained will depend on the quality of study-what Boyd (1972) called "cage-side observation." From the standpoint of accidental exposure to pesticides, one of the most useful pieces of information is the comparison between the dermal and oral acute lethalities. Because occupational exposure to pesticides is largely by the dermal route, those pesticides that are absorbed through the skin in sufficient amounts to kill animals that receive small dosages definitely represent more of a potential hazard to people than materials that are not absorbed through the skin in toxic amounts except at high dosages.

The one-dose ED 50 or LD 50 has served a useful purpose in defining the approximate toxicity of chemicals with almost no theoretical justification for the way such experiments were and still are conducted. The usual protocol entails the administration of a compound by some route of exposure and then to determine the effect (death) after a specified length of time, which usually is 14 days. There are numerous and severe problems with this protocol from the theoretical point of view. The 14-day observation period is entirely arbitrary and lacks scientific rationale. It is the time response at constant dose that determines the length of the observation period needed after a single dose Rozman (1999). It can be 70 days (e.g. HCDD-induced wasting) or just a few minutes (e.g. CO-induced asphyxiation) depending on the time to effect (death). Similarly problematic is the counting of dead animals. As strange as it might sound, one dead animal may not be the same as another dead animal if the two died by a different mechanism of action which is often the case after supralethal doses when recovering animals still succumb to a damage not repairable during the allotted observation period (e.g. 14 days). For example, rats exposed to NO_2 acutely by inhalation die of either spasm of the larynx or edema of the lungs. Spasm of the larynx and lung edema have very obviously different mechanisms of action and as such are part of different dose and time responses. Therefore, they will distort the $c \times t$ concept if lumped together.

The biggest problem with the ED 50 and LD 50 studies is that for the most part they were and are not conducted under conditions of toxicodynamic and/or toxicokinetic steady state and, therefore, almost all studies measure not only toxicity, but toxicity and recovery at the same time. Depending on the ratio between recovery/elimination half-life and the observation period, the dose response will be increasingly distorted in the form of a flattening of the sigmoid curve. Under very unfavorable experimental conditions when very little toxicity versus a great deal of recovery is being measured the lower part of the S-shaped curve appears nearly linear with dire consequences for the accuracy of any risk assessment based on such faulty interpretation.

Shape of the ED 50 or LD 50 Curve Several matters regarding determination of ED 50 and LD 50 values are illustrated by Fig. 1.4 based on LD 50 studies of DDT. All parts of the graph represent the results of tests in which the groups of rats were given various dosages of the compound. In part A, the dosage for each group has been plotted on plain graph paper against the percentage of mortality. The fundamental statistical principles illustrated by part A first were defined clearly by Trevan (1927). Specifically, he pointed out that there was no such thing as a minimal lethal dosage or minimal effective dosage conceived at the time, namely, a dosage that would be just sufficient to produce the effect in all animals of a given species. He noted that the variability of individuals in a population led to the characteristic S-shaped curve and that there seemed to be less variability at the 50% level of response. Trevan proposed the equivalent terms "median lethal dose" and "LD 50," both in their presently accepted meaning. He also suggested that dosages that kill other proportions of large groups of animals be designated by analogous symbols, for example "LD 25" and "LD 75" for dosages that kill

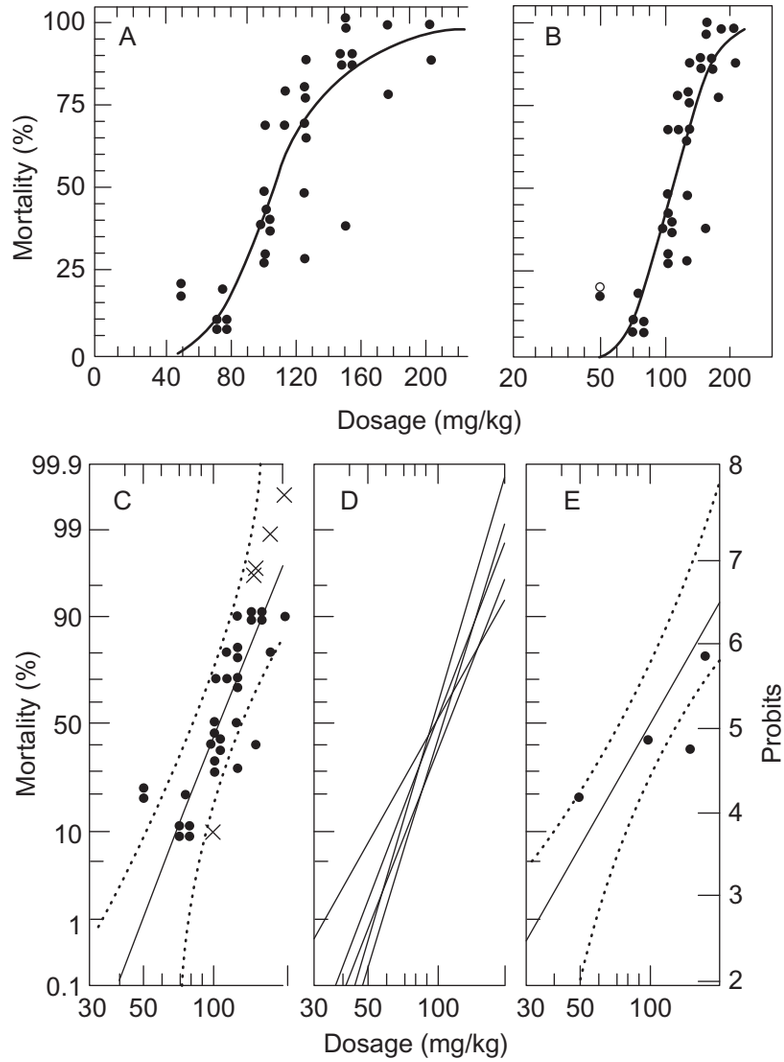

FIGURE 1.4 Mortality of white rats caused by oral doses of DDT. Each point represents one group of animals, usually 10; (A) percentage mortality in six separate tests plotted against dosage; (B) the same data with percentage plotted against logarithm of dosage; (C) the same data with percentage mortality expressed as probits plotted against logarithm of dosage; the 19/20 confidence limits are shown by dotted lines on either side of the dosage–response curve; (D) dosage–response curves for each of the six separate tests; (E) the dosage–response curve that differed most from the others, showing its relatively wide confidence limits.

25 and 75% of the group, respectively. In Trevan's paper in the *Proceedings of the Royal Society,* the symbols were printed with a space between the letter "D" and the appropriate number. This style has been adopted for this chapter, partly because it is authentic and partly because it is completely clear when it becomes necessary in theoretical discussions to refer to fractions of a percentage, for example, "LD 0.01."

The LD 50 can be read from the curve even in its S-shaped form on plain graph paper. Thus, in part A, the level for 50% mortality intersects the curve at a dosage of about 113 mg/kg. It may be noted parenthetically that the middle portion of the sigmoid curve-in the region of 20–80% response is often indistinguishable from a straight line. The fact that a simple straight line relationship between dosage and percentage response adequately describes some sets of data must not obscure the fact that more complete data determine a sigmoid curve on plain paper.

Part B of Fig. 1.4 represents the same data shown in part A, but dosage is now shown on a logarithmic scale rather than on a simple arithmetic scale. The S-shaped pattern persists, but the curve approaches a straight line. Part C of the graph represents the same data plotted with an additional conversion. Here the logarithm of dosage is plotted against percentage mortality expressed as probits. The correspondence between percentage and probits is shown

by the scales on the left and right of the lower portion of the graph. As may be seen, the points are scattered about a straight line when the full (logprobit) conversion is made. The logarithmic conversion apparently was introduced first by Krogh and Hemmingsen (1928), but it was the subject of numerous publications cited by Gaddum (1933) in the classical paper in which he introduced the full conversion essentially in the form still used today. Actually, Gaddum employed normal equivalent deviations rather than the probits that are commonly used today. However, a probit is merely a normal equivalent deviation to which 5.0 has been added for convenience to eliminate negative values. The paper by Gaddum was shortly followed by three papers by Bliss (1934a,b, 1935a). These four papers on the statistical relationship between dosage and response are still basic today. The facts regarding probit analysis were summarized in a masterful way by Finney (1971) in a book first published in 1947 and revised in 1971. A more general treatment of the principles of biological assay is that of Emmens (1948).

It must be stated that the slender sigmoid curve we have discussed constitutes (insofar as data permit one to judge) a cumulative lognormal curve. Conversion of the percentage response to normal equivalent deviations or probits is merely a statistical device for converting the sigmoid curve into a straight line. Although Gaddum (1933) employed the logarithmic conversion as well as the normal equivalent deviation conversion for use in toxicity measurements, it apparently was not until 1945 that he introduced the word "lognormal" to describe the situation in which log x is normally distributed. In the same paper, Gaddum (1945) emphasized that the distribution of values for many parameters in nature is not statistically normal. This means only that the distribution frequently does not conform to any of the family of curves commonly called *normal* or *Gaussian* after Karl Friedrich Gauss, who first popularized this particular pattern of variation. Use of the word "normal" in this connection has no bearing on physical or biological normalcy. In fact, Gaddum pointed out that if the distribution of the volume of particles is normal the distribution of their diameters will, of necessity, not be normal.

Gaddum stressed the importance of converting measurements in such a way that the results may be subjected to statistical evaluation. In addition to logarithmic conversion of each variable for this purpose, he suggested that a positive or negative constant might be added to each variable prior to its logarithmic conversion.

The logprobit conversion has great value for purposes of description and statistical analysis. However, in spite of its great practical value, the basic assumption that the relationship of variables is perfectly lognormal cannot be considered proved, because the upper and lower extremities of the curve have not been studied experimentally to a sufficient degree. This detail is discussed in Section 1.3.7.4.

The shape of the dose-response curve has been the subject of vigorous discussions because of its utmost importance for risk assessment. Statistical approaches for its description abound but a theoretical treatment of it had not been proposed until Rozman *et al*. (1996) suggested, as a first simplified approach, a Malthusian-type statement of a problem described by the Verhulst equation addressing the issue of change under constraint. The constraint in toxicology is the impossibility of having more than 100% of a population affected. Thus, the effect as a function of dose or time is always proportional to the effect that has already occurred as well as to the effect still remaining to occur. The Verhulst equation has an exponential solution in terms of effect and not in terms of dose, which is compatible with the notion that the effect in a population has logarithmic properties (normal distribution) but the dose (number of molecules) does not have such properties. However, the dose-response has been traditionally plotted on a log (dose) vs effect (arithmetic) scale. Because log (dose) vs effect is the inverse function of log (effect) vs dose (arithmetic) and because inverse functions are entirely symmetrical, there has been no problem with this plot, even though in terms of epistemology the traditional way of plotting the dose-response function may not necessarily be entirely correct. It must be emphasized, that nonlinearity of the dose-response can also be derived from thermodynamic considerations as well (Rozman, 2003 a,b; Waddell, 2008).

Under ideal conditions of toxicodynamic or toxicokinetic-toxicodynamic steady state any dose response is extremely steep, best exemplified by inhalation anesthesia (see Storm and Rozman, 1998). There is a factor of no more than 2 between doses, which will anesthetize the most and least sensitive individual. Inhalation of a volatile agent is kinetically related to intravenous infusion, which for compounds of short half-life will provide steady state concentrations very rapidly. Similarly, the dose causing 100% wasting-hemorrhage and 0% of this effect in rats under conditions of toxicokinetic steady state is also a factor of 2 (Rozman, 1999). Similar considerations are valid for compounds that act by toxicodynamic mechanisms. Departure from either type of steady state condition will introduce recovery as an additional variable. The vast majority of toxicological experiments (and real-life situations) are not conducted or do not occur under steady state conditions. The resulting introduction of one or more variables in addition to toxicity is the reason for the large variability in interlaboratory experiments and the mistaken assumption of flat dose-responses. Under ideal conditions, when all variables other than toxicity are controlled, all dose-responses are as steep as the ones discussed previously.

ED 50, ED 1, ED 99, and Corresponding LD Values

Returning to part C of Fig 1.4, it may be seen that the level of 50% mortality intersects the curve at a dose of 113 mg/kg.

This is the oral LD 50 for DDT as indicated by the observed data. In a similar way, the 1% mortality level intersects the curve at a dosage of 52 mg/kg, which, therefore, is the LD 1. Mortality of 99% is not shown on the graph but would fall at a dosage of 223 mg/kg, which is the LD 99.

Confidence Limits and Reproducibility The degree of scatter of observed values may be evaluated by calculation and expressed as a confidence limit. These limits are shown by dotted lines on both sides of the solid line in part C of the graph. These particular confidence limits indicate the area or range within which the dosage-response line may be expected to fall in 19 of 20 samples taken at random from the same population. It may be seen that a series of such curves will correspond closely with one another at the 50% mortality level but will agree less well as the mortality approaches either 0 or 100%. This is a graphic representation of the fact first noted by Trevan (1927) that the LD 50 may be estimated more accurately than corresponding statistics for greater or lesser effect (e.g., LD 99 or LD 1). This is also true of ED 50 values and corresponding ED 99 and ED 1 values.

The points represented in parts A, B and C of Fig. 1.4 represent the results of six separate tests made in six different years for the purpose of determining whether there was any change, genetic or otherwise, in the susceptibility of the particular colony of rats to acute poisoning by DDT. Part D of the graph shows the dosage-response lines determined in connection with the six separate tests. The lines correspond very closely at the 50% mortality level but diverge somewhat in connection with higher or lower mortality rates. Actually, all of the lines are in good agreement, indicating that there was no detectable change in the colony concerning susceptibility to DDT. In fact, the dependability of this kind of test is well recognized. Weil *et al*. (1966) reported that they had done one-dose oral LD 50 of 26 chemicals annually for 11 or 12 years to determine the reproducibility of the test and the dependability of commercial production of the chemicals. The resultant median lethal doses were relatively unaffected by the different annual samples of each chemical, by changes in the stock or rats, by the degree of dilution of the toxicants, or by change in the personnel performing the tests. Only one variable, the weight of the rat, appeared to have a significant effect on the values obtained, which is consistent with a report of Lamanna and Hart (1968) as interpreted in Section 1.5.1 by Rozman and Doull.

Slope and Its Relation to Confidence Limits Part E of Fig. 1.4 shows the data and resulting curves for a single LD 50 determination, namely, the particular test that differed most from the average of the six tests. It may be seen that the slope of the line is greater than the slopes of the other LD 50 lines (part D). This increase in slope

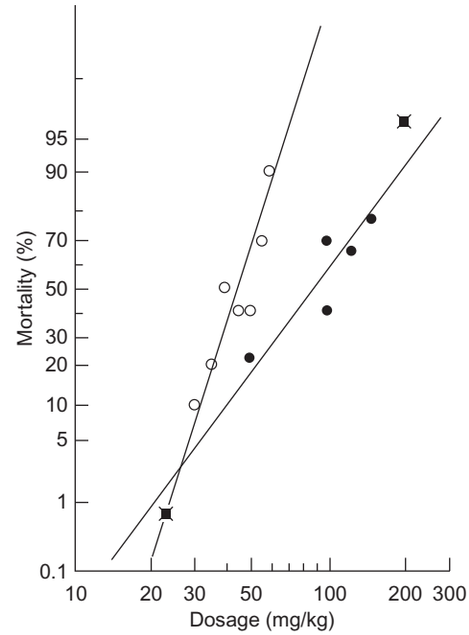

FIGURE 1.5 Dosage–response curves for dieldrin (o) and toxaphene (●) given orally to white rats. Points adjusted according to the method of Litchfield and Wilcoxon (1949) are distinguished by a superimposed x.

is a graphic representation of the data on which this particular determination was based. The greater variability of the data is also reflected by the fact that the dotted curves (representing confidence limits) lie farther from the solid line than the corresponding curves do in part C of the figure.

Procedures for Determining ED 50 and LD 50 Values Parts C and E of Fig. 1.4 represent actual determinations of LD 50 values using the graphic method proposed by Litchfield and Wilcoxon (1949), who also supplied details of the method for calculating the 19/20 confidence limits. A number of non-graphic methods are available for determining ED 50 or LD 50 values, including the methods of Bliss (1935a,b, 1938). The non-graphic methods have in common the fact that percentage values must be transformed by means of an appropriate table or calculation. A wide variety of methods have been reviewed by McIntosh (1961), who concluded that the differences among results with the 15–85% response range are negligible. Thus, selection of the method to use depends largely on personal choice.

Repeated determinations of an ED 50 or LD 50 for a particular compound under the same conditions should give not only statistically indistinguishable values but also statistically indistinguishable slopes of the dosage-response curves as shown in Fig. 1.5. The curves may be related in such a way that the ED 50 (or LD 50) values are statistically distinguishable but other values such as the ED 1

(or LD 1) are not distinguishable. Figure 1.5 offers an example. The LD 50 values for dieldrin and toxaphene are different but the LD 1 values for these compounds are statistically indistinguishable.

Hayes was well aware of the problems arising from a lack of control of variables. He, like others, proposed a statistical treatment of data to deal with "hidden" variables, which cannot be readily identified. This was appropriate at the time he wrote this chapter. However, even a partial development of a theory of toxicology requires a different type of approach. The question is not how to accommodate the apparent difference in the slope of the dieldrin and toxaphene mortality dose responses statistically. Rather the question is why is the toxaphene dose response slope flatter than that of dieldrin (Fig. 1.5). The half-life of dieldrin in rats is estimated in the range of weeks (Mueller *et al*., 1975) whereas that of toxaphene is in the range of 4–7 days (ATSDR, 1996). Therefore, during an observation period of 14 days little of dieldrin will be eliminated whereas most of toxaphene will have been excreted. Consequently, not much recovery takes place with dieldrin during the observation period (14 days), whereas a great deal of recovery occurs with toxaphene combined with the concurrent development of toxicity. It must be understood that just because some animals eventually succumb does not mean that they did not try to recover from the damage if time for recovery was available relative to the half-life of the compound or of the effect. If toxaphene had been administered in a way to make its kinetics similar to that of dieldrin (loading dose rate followed by maintenance dose rates) then the two dose responses would have been parallel (identical slopes). This is an extremely important issue. It is clear that dieldrin is a more toxic compound than toxaphene, which is also indicated by Fig. 1.5 when comparing the highest doses of these two chemicals. However, comparing the lowest doses suggests that at low doses toxaphene is equally as toxic as or more toxic than dieldrin. This is of course impossible because relative potency is an intrinsic property of a chemical. In fact, this illusion of dose-dependent relative potency arises as a result of a lack of experimental control of the kinetics of toxaphene, whereas that of dieldrin is controlled coincidentally by its long half-life. The lack of kinetic control is very frequently one of the "hidden" variables in toxicologic experiments. Similarly, a lack of control of absorption after dermal application of chemicals can be only partially controlled by standardizing dosing volumes. Applied dermal dose will still remain a poor surrogate for systemic dose in most instances as discussed in Section 1.3.6.

Test Using Small Numbers of Animals The conventional procedures for determining ED 50 or LD 50 values require the use of approximately 50–100 animals. The use of this many rats or mice may be practical, even though somewhat expensive. The use of a similar number of dogs or monkeys often is entirely impractical. To meet this problem a number of methods have been developed that permit the use of a small number of animals per group to determine approximate ED 50 or LD 50 values (Gaddum, 1933; Deichmann and LeBlanc, 1943; Weil, 1952; Smyth *et al*., 1962).

Volume of Each Dose If the results of toxicological tests are to be compared, it is wise to keep all conditions as nearly uniform as possible. One variable that should be standardized is the volume of solution or suspension in which compounds are administered. It has been found practical to give most oral doses at the rate of 0.005 ml per gram of body weight and to give dermal applications at the rate of 0.0016 ml per gram of body weight. Differences in dosage are determined by changing the concentration. The value of 0.0016 ml/g was chosen for dermal application because it represents a plausible exposure of about 100 ml for a human and also gives even numbers for dosage associated with many formulations actually used in the field. Thus, at this rate of application, the dose for a 70-kg human would be 112 ml – a not unlikely degree of contamination as the result of spillage. Formulations of 0.312, 0.625, 1.25, 2.5, 5, and 10% produce dosages of 5, 13, 20, 40, 80, and 160 mg/kg, respectively, when applied at the rate of 0.0016 ml per gram of body weight.

1.3.1.2 90-Dose ED 50 or LD 50

It has been suggested, more or less empirically, that subacute tests should occupy up to one-tenth of the life span of the experimental animals (commonly considered to be about 90 days for the rat and 1 year for the dog) [Food and Agriculture Organization/World Health Organization (FAO/WHO), 1958].

Boyd (1961) accepted the concept of one-tenth of the life span, but considered it to be 100 days in the rat. However, the important thing is not the choice or definition of a particular fraction of the life span but the selection of a testing interval that is as short as practicable and yet will give meaningful information about the effect of absorbing the toxicant during an entire lifetime. Secondarily, it would be desirable to have a standard test so that results from different laboratories would be reported in the same terms.

Apparently Boyd and Boyd (1962) were the first to report subacute toxicity in the form of an LD 50. The compound was administered intramuscularly for as long as 100 days. In connection with oral doses it was proposed (Boyd and Selby, 1962) that the compound under test be administered by stomach tube for 100 days. The test differed in some technical requirements from the 90-dose test described subsequently. In spite of this, the two tests are fundamentally similar, and the results of one are largely interchangeable with those of the other.

Several years after he had proposed the 100-day test, Boyd (1968) pointed out that, for compounds he studied,

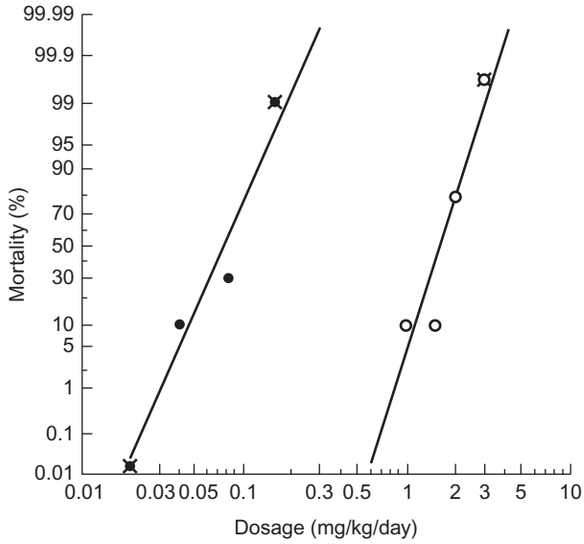

FIGURE 1.6 Oral dosage–response curves for 1-dose (o) and 90-dose (•) tests of warfarin. Points adjusted according to the method of Litchfield and Wilcoxon (1949) are distinguished by a superimposed x. From Hayes (1967b), by permission of Academic Press.

TABLE 1.1 Toxicity of Warfarin to Male Rats[a]

	1-Dose	90-Dose
Number of rats tested	50	110
Survival time (days)	5–10	3–43[b]
LD 50	1.6	0.077
19/20 confidence limits[c]	1.4–1.9	0.055–0.108
Lowest dose to kill[c]	1.0	0.04[d]
LD 1[c]	0.84	0.032

[a]From Hayes (1967b), by permission of Academic Press.
[b]The true range may be 3–25 days; warfarin was probably not the cause of death in the rat that died after eating the compound for 43 days.
[c]Expressed as milligrams per kilogram for 1-dose test or milligrams per kilogram per day for 90-dose test.
[d]This death probably was not caused by warfarin. The smallest dosage to cause a death clearly related to warfarin was 0.08 mg/kg/day.

the test could be reduced to about 70 days with little or no loss of important information.

Weil and McCollister (1963) showed that the results of 90-day studies not only in rats but even in dogs were similar to corresponding lifetime studies in these species for a wide range of compounds.

Hayes (1967b) pointed out that a 30-day test in the rat would be adequate for some compounds (e.g., potassium cyanide).

However, a review of data on certain chemosterilants showed that, although 30 doses were entirely inadequate to reveal the potential injury caused by repeated doses of any of them, 90 doses gave for most of them essentially the same results as those of tests lasting twice as long. Thus, although a test involving fewer than 90 doses would be adequate to reveal the effect of long-term exposure to many compounds, a 90-dose test is more generally valid for predicting lifetime effects. In fact, even a 90-dose schedule is inadequate to define the long-term toxicity of some compounds such as hempa (hexamethyl phosphoric triamide); however, it was clearly evident after 90 or even somewhat fewer days that animals being dosed with hempa were still dying and that the exposure would have to be prolonged to assess the toxicity properly. Thus a standard of 90 doses was selected for quantitative study of the effect of repeated doses, partly because 90-dose tests were already widely accepted for other purposes and in spite of the limitations just mentioned.

The 90-dose ED 50 (or 90-dose LD 50) is statistically comparable to a 1-dose ED 50 (or 1-dose LD 50). In both tests, percentage effect expressed as probits is related to dosage expressed as logarithms.

The results of a 1-dose and a 90-dose LD 50 study of warfarin are shown in Fig. 1.6. It may be seen that the curves are similar in slope although the dosages that proved critical differ by a factor of about 20. Table 1.1 shows the LD 50 values and related statistics for warfarin.

Hayes (1991) as well as others struggled between practicality and awareness of the importance of time in multiple-dose-rate experiments (subacute, subchronic). Unfortunately, a theory leaves no room for considerations of practicality because laws of nature exist on their own timescales with complete disregard for human convenience. As was the case for acute studies with the 14-day observation period the timescale of the 90-dose-rate studies is largely arbitrary. Naturally, a number of effects will become manifest in 90 days (plus an additional 14-day observation period for recovery) that cannot be seen in 14 days. Nevertheless, the selection of 90 days or 104 weeks is as arbitrary as the 14-day observation period after single dose rates or after the 14-day off-dose observation period at the end of a subchronic experiment. A similarly arbitrary timescale is the l04-week carcinogenicity bioassay. Having recognized time as a quantitative and quantifiable variable of toxicity together with the need for mechanistic definition of an effect requires that each effect must be studied on its own timescale. Although the 90-dose-rate study may be still retained for the time being as a rough first estimate of potential sub-chronic effects, mechanistic studies should be conducted on the timescale of a given effect. For example, sub-chronic warfarin-induced hemorrhagic death does not need to be studied in 90-dose-rate studies because no treatment-related lethality occurred after day 25 (Hayes, 1967b). Clearly recovery in the form of adaptation has taken place. Any further sub-chronic studies should have been conducted on a timescale no longer than 25 or perhaps 30 days, if the population studied was no larger than that used by Hayes (1967b).

Determination of the 90-Dose ED 50 or LD 50 In calculating the conventional 1-dose LD 50, no account is taken of time, although, of course, animals given a single dose do not respond to it simultaneously. The 90-dose ED 50 is managed in a similar way; the animals are held long enough after the last dose to be sure that all reactions have been counted. The two procedures differ, because in the 1-dose test all animals in a group receive the same dosage, but in the 90-dose test animals in the same group may receive different total dosages because some may survive longer than others. This difference does not invalidate a comparison of the results of the two tests, but makes quantification difficult (Saghir *et. al.,* 2005).

In determining the acute oral ED 50, the compound is usually administered by stomach tube. In determining the oral 90-dose ED 50 the compound is administered as a mixture in the diet. This difference in technique for oral administration introduces a second kind of difference (applying to this route only) between the two kinds of oral ED 50 values, but it has two advantages: convenience and realism. Obviously it is more convenient to maintain an animal on a special diet for 90 days than to dose the animal by stomach tube for the same period. Except in connection with drugs, which are administered in discrete doses, it is also more realistic to administer repeated doses in the diet rather than by stomach tube. If people receive relatively regular repeated doses of an environmental compound, the intake is usually distributed throughout a considerable portion of most days while the result of a single massive exposure due to a splash or other spillage associated with occupation, or the ingestion of a relatively concentrated material due to accident or suicide. Furthermore, if a number of compounds are administered by any particular route in such a way that the absorption of a single dose of each is concentrated in as short a period as possible, but the absorption of repeated doses of each is distributed as evenly as is practical over each day, then any difference in cumulative effect of the compounds will be demonstrated to greatest advantage.

Thus, to determine the oral 90-dose ED 50 (or LD 50) of a compound, appropriate concentrations in ground chow are fed to groups of animals for 90 days. All survivors are then fed chow without the compound for a minimum of an additional 2 weeks, and, if any of them are still affected, they must be observed for as long as necessary until they have died or recovered. The dosage (expressed as milligrams per kilogram per day) is calculated from measured food consumption (Hayes, 1967b).

Although Hayes (1991) and others were aware of the importance of time, time was dealt with only semiquantitatively in toxicology. It is true that in the single-dose-rate studies the animals receive the same dose whereas in multiple-dose-rate experiments they may receive different doses because animals die at different times. It is also correct that this does not invalidate the comparison between them because the concentration at the site of action (steady state) is the critical variable, for which the dose or dose rate is just a surrogate measure.

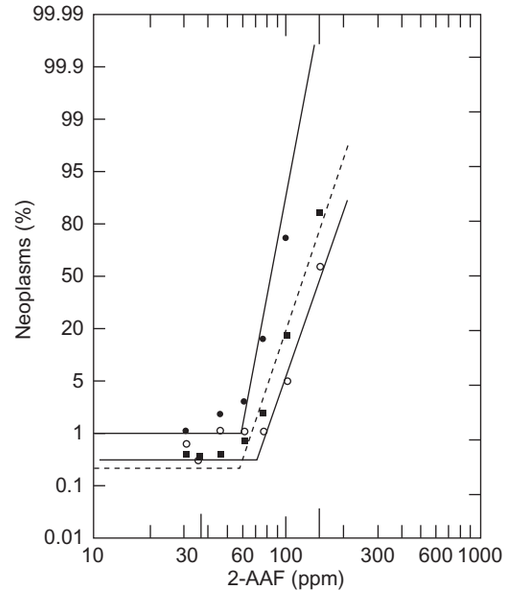

FIGURE 1.7 Bladder neoplasms in dead, moribund, and sacrificed mice fed 2-AAF continuously: (●) 33 months; (×) 24 months; (O) 18 months. The graph is based on data by Farmer *et al*. (1980).

Administering a compound by gavage in the 1-dose-rate studies or mixed with the diet in the 90-dose-rate studies again has a practical basis. The absorption phase after gavage provides for a longer near steady state condition for compounds having half-lives of about a day or longer. Feeding a compound to rats also improves kinetics in the course of subchronic experiments, because rats have two or more feeding periods per day, which provides more nearly steady state conditions for compounds of intermediate half-life than does a single dose rate per day by gavage. It is unfortunate that the widely used study designs (acute, subchronic, chronic) in toxicology were developed with little or no consideration of kinetics. This is understandable, though, in historical context because study designs of toxicological experiments were already firmly ingrained by the time the first book was published on pharmacokinetics (Dost, 1953). Nevertheless, no significant advance in theoretical toxicology can take place until study designs are changed to accommodate toxicodynamic and/or toxicokinetic time scales as quantitative and quantifiable variables of toxicity.

1.3.1.3 The ED 01 and Related Studies

Logprobit analysis may be applied to any study of dosage response regardless of the duration of dosing or the effect that is recorded. The following paragraphs outline this kind of analysis of two studies of cancer. The theoretical basis for using logprobit analysis to investigate the fundamental problem of small dosages is discussed in Section 1.3.7.4.

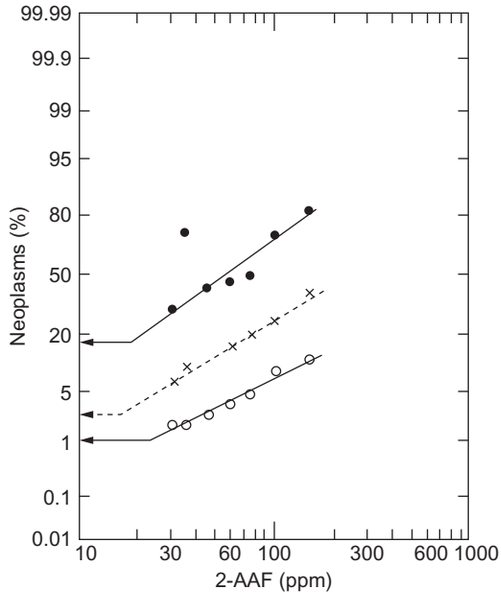

FIGURE 1.8 Liver neoplasms in dead, moribund, and sacrificed mice fed 2-AAF continuously: (●) 33 months; (×) 24 months; (○) 18 months. The graph is based on data presented by Farmer *et al*. (1980).

The ED 01 Study As of 2009 there had been only one statistical study for which the raw data were easily available of the effects of small dosages of a chemical. In this ED 01 study (Staffa and Mehlman, 1980; Hughes *et al*., 1983), a predetermined number of mice at each dietary level were killed, after being fed continuously at different dietary levels of 2-acetyl-aminofluorene (2-AAF), at intervals of 9, 12, 14, 15, 16, 17, 18, 24, and 33 months.

One paper in the original report (Farmer *et al*., 1980) presented the raw data for tumor incidence and also presented logprobit graphs of the incidence of bladder tumors as related to dietary concentration for the 18-, 24-, and 33-month intervals. A similar graph was presented for the incidence of liver tumors. These two graphs were certainly unusual in that the points on which they were based were not shown. Corresponding graphs have been prepared that differ in two ways: the observed points have been plotted and the control values have been noted. Values that could not be plotted on probit paper-because the expected values are too small or too large to be subject to correction according to the method of Litchfield and Wilcoxon (1949)-have been shown by arrows indicating the direction at which the points would lie at infinite distance to the left on logarithmic paper. The graph for the bladder (Fig. 1.7) confirms what the original authors admitted, that is, that the curves for different time intervals are consistent with the view that there is a threshold dosage below which 2-AAF does not increase the incidence of bladder tumors above that seen in controls. In other words, the curves remain straight until they reach the area of the graph where the incidence values are indistinguishable from those observed in the controls. It could be argued that some of the values at the lower part of the curve tend to be displaced upward and to the left. However, this interpretation is unjustified because the incidence of tumors in the control group showed no trend in terms of time. As a matter of fact, the highest incidence was observed at the first interval, that is, a value of 1.47% at 9 months. Thus, the entire range of incidence observed among the control groups must be considered. If this is done, the points that might otherwise be interpreted as occurring above and to the left of the straight line are seen simply to be indistinguishable from control values.

Farmer and his colleagues (1980) argued that the graph for liver tumors completely excluded the possibility of any threshold. In the summary and conclusions (Gaylor, 1980) it was stated that "liver tumors showed a nearly linear response over the experimental dose range, thereby dispelling any notion of a threshold dose." One would have to agree with this statement if it was intended to mean that the results ruled out a threshold at any one of the dietary levels of 30 ppm or higher. However, Fig. 1.8 shows that the curve did closely approach the control levels and suggests that, if a wider range of dosages had been used, the threshold might have been encountered in the range of 16–23 ppm or, more conservatively, in the range of 10–30 ppm.

The first report on the ED 01 study is notable for neglecting the control values and, more broadly, for ignoring the scientific question of whether the values observed above the noise levels determined by the controls were consistent with a cumulative lognormal distribution.

The report of the workshop held in September 1981 (Hart *et al*., 1983) was apparently concerned mainly with integrating time with dosage-response. It was concluded (Hughes *et al*., 1983) that "the ED 01 Study demonstrates the observed risk is more adequately expressed in a time and dose continuum rather than simply as dose." The conclusion undoubtedly is correct and was supported by very sophisticated calculations. However, the question of whether the observed increase in incidence above control values was cumulative lognormal in distribution was neglected. The conclusion was that "even with a study as large as the ED 01 study, statistical uncertainty makes it impossible to establish the true shape of the dose-response curve at low tumor rates. Neither can such studies prove or disprove the existence of thresholds."

The ED 01 study, also called the megamouse experiment, is indeed very important for toxicology. It provides several lessons beyond those identified by Hayes (1991). The ED 01 study was designed with a lack of kinetic considerations but the initiators were lucky that the dynamic half-life of 2-AAF-induced damage is such that feeding it in the diet provided a steady state of injury (Rozman *et al*., 1996; Rozman, 2000b). It was also fortuitous that there was not enough toxicity as the experiment progressed

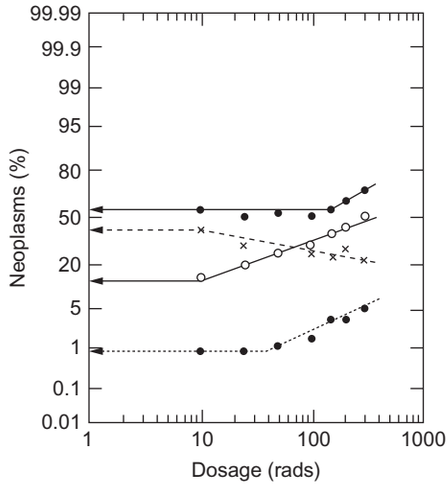

FIGURE 1.9 Incidence of thymic lymphoma (○), myeloid leukemia (◌), reticular cell sarcoma (×), and totalreticular cell neoplasms (●) in mice that received different dosages of gamma radiation. The graph is based on data presented by Ullrich and Storer (1979a).

and therefore the 24- and 33-month sacrifice schedules were added. Otherwise a reconstruction of the liver dose response from the time response would not be possible.

There was much greater emphasis on statistical considerations than on toxicological theory in the design of this very large experiment. However, it is remarkable how little attention was paid to Druckrey's $c \times t$ studies on cancer (Druckrey *et al*., 1963; Druckrey *et al*., 1967). The interpretation of the study was also driven by statistics and not by the science of toxicology. Revisiting the megamouse study revealed that the occurrence of both bladder cancer and liver cancer was highly consistent with the thesis of Druckrey that $c \times t = k$ (Rozman *et al*., 1996). For the controversy about linearity or nonlinearity of the dose response there is a straightforward biological explanation. 2-AAF is a more potent bladder carcinogen and a less potent liver carcinogen. Therefore, the ED 01 study generated a fairly complete dose response with a shallow and a steep part in terms of bladder cancer with an identifiable threshold. Both the dose-response and the time-response for liver cancer occur to the right of the corresponding bladder cancer response, mainly toward the end of the animal's life span. Therefore, in terms of liver cancer only the shallow part of the dose response is documented by data, because the steep part of it was prevented from developing by the natural life span of the mice. Thus, the low dose linearity is an illusion, which is inconsistent with the theory of toxicology that under conditions of toxicokinetic or toxicodynamic steady state at constant time all dose responses must have also a steep part of their slope. However, if the time-response of a particular effect is truncated by the life span of the species then it is impossible to establish a complete dose response for that particular end point of toxicity. Nevertheless, it is possible to construct a hypothetical dose-response curve beyond the natural life span of a species using the $c \times t = k \times W$ conversion. Such a hypothetical dose response for 2-AAF becomes also very steep for liver cancer beyond the natural life span of mice. It is important to note that this biological interpretation of the ED 01 study is entirely consistent with statistical approaches as exemplified by the Hartley-Sielken model using both dose and time as variables of toxicity (Hartley and Sielken, 1977a,b), but is completely at odds with currently used linear extrapolation models using only dose as a variable of toxicity.

Dosage-Response to Radiation At least one study involving a very large number of animals exposed to varying doses of gamma radiation is available (Ullrich and Storer, 1979a,b,c). Including controls, there were 17,587 mice, of which 15,558 were female. All graphs were on plain graph paper, with incidence of tumors plotted against dosage in rads. No attempt was made to explore the logprobit relationship, an omission that, because Gaddum's famous paper was published in 1933, appears unjustified. Because Ullrich and Storer presented raw data, it is possible to explore how their results fit the cumulative lognormal concept. The curves for reticular cell neoplasms in female mice are shown in Fig. 1.9. Here again, the results for thymic lymphoma and for myeloid leukemia are entirely consistent with a linear logprobit relationship that intersects the control level. However, the graph also indicates that, at least within the range of 25–150 rads, increasing doses of radiation caused a decrease in the incidence of reticular cell sarcoma with the result that the total number of reticular cell neoplasms did not begin to increase until the dosage exceeded 100 rads. The authors discussed this phenomenon but failed to consider whether the decrease of one kind of tumor and the increase in other kinds of tumors were independent phenomena or whether the reticular cells that otherwise would become sarcomas were somehow converted by radiation to malignant cells or other configurations that appeared as thymic lymphomas and/or myeloid leukemias. Not shown is a graph for solid tumors in the same mice (Ullrich and Storer, 1979a,b,c). The incidence of ovarian, pituitary, and Harderian gland tumors in excess of controls was consistent with the cumulative lognormal concept. The incidence of lung adenomas was somewhat less at dosage levels of 10–150 rads than it was in the controls, but there was no clear-cut dosage-response relationship such as that for reticular cell sarcoma.

Radiation-induced toxicity is a classical, limiting case of toxicology. It is a hit-and-run type poison, whose effects are entirely determined by the dynamics of injury and by exposure frequency. Because the toxicokinetic half-life (residency time of radiation in an organism) is close to zero and because $e^0 = 1$, radiation is independent of toxicokinetics

(unless delivered by a carrier such as radon), which simplifies the equation of toxicity to

$$\frac{dT}{dE} = \frac{dT}{dD} \cdot \frac{dD}{dE}$$

Moreover, because radiation-induced injury is extremely rapid it must be recovery from injury that dominates the dynamics of radiation toxicity. In fact, recovery rate constants have been calculated for radiation-induced injury (Sacher *et al*., 1949; Sacher, 1950 cited in *Radiation Biology 1954),* although quantitative predictions were deemed to be problematic. One of the problems was that recovery was nonlinear (Steamer and Christian, 1951), which would have required more data points and curve stripping to separate adaptation and repair rate constants. The other problem was the lack of a clear experimental design to keep all but one timescale constant, when studying a particular effect. The lack of precise diagnostic causes of death introduced further "hidden" variables in terms of the effect (Steamer, 1951). These are the main reasons for not finding robust quantitative predictions in radiation-induced toxicity. It needs to be reiterated that quantitative $c \times t$ or $c \times t^x$ relationships can be seen only under ideal conditions that is lack or very slow reversibility or continuous exposure to effect and/or regular departures from it (see Section 1.1.3).

Discussion The conclusion that large scale animal studies can neither prove nor disprove the existence of thresholds does not really depend on statistical uncertainty, but on our uncertainty in understanding the basis for any thresholds that exist. We never really accept conclusions that we do not think we understand. As discussed in Section 1.3.7.4, the basis for so-called understanding may vary all the way from very detailed biochemical information (as illustrated by our understanding of the value of vitamins and essential trace elements) to mere economic result (as illustrated by the benefits of food additives for certain livestock).

It is not possible to claim the theoretical existence of a threshold on considerations of dose alone because a single molecule in an infinitely large population or in a finite population with eternal life could cause an effect. However, it is very straight forward to define a threshold for an effect using both dose and time as variables of toxicity because the maximum-life-span-dose combination allows calculation of a practical threshold dose which will have no effect whatsoever in a lifetime in any defined population size (Rozman *et al*., 1993; Rozman *et al*., 1996; Rozman and Doull, 1998; 1999; 2000).

1.3.1.4 Kinds of Phenomena Showing a Cumulative Lognormal Form in Their Dosage-Response Relationships

As reviewed in the foregoing sections, the pharmacological and lethal effects of compounds on intact organisms have the form of cumulative lognormal curves, which can be plotted as straight lines following probit conversion. In biology, the log-normal curve is used rarely except in measuring the responses of intact organisms. However, similar curves are obtained when the concentration of a compound is plotted against the inhibition it causes in the activity of an enzyme. The dissociation curves of oxyhemoglobin also have a similar form (Gaddum, 1937). The fact that this form of dosage-response relationship is found in connection with tissues, enzymes, and macromolecules indicates its fundamental nature.

In fact, when the initial concentration (or dosage) of one kind of molecule (e.g., a toxicant) is plotted against the percentage of these molecules reacted with another kind of molecule (e.g., an enzyme or macromolecule) present in excess, the resulting graphs are statistically indistinguishable from straight lines within the range of 10–90% reaction. At the extremes, that is, below 10% and above 90%. deviation from linearity is observed. Such effects can be modeled using simple expressions derived from the law of mass action or, more appropriately expressions derived from a consideration of cooperative ligand binding (e.g., the Hill equation). The concept of a threshold can be incorporated into either model. However, a truly realistic model must include kinetic consideration of the rate of inactivation of the poison and the repair of the biochemical lesion. Certainly, this realistic modeling could be complex. However, it might be useful in defining the quantitative aspects of remaining problems. Of course, it is already known that small concentrations of a toxicant may be withstood by the intact organism because it can tolerate some inactivation of enzymes and macromolecules and because critical molecules are replaced in the course of normal repair. Known examples of a chemical basis for thresholds in dosage-response relationships are discussed in Section 1.3.7.4.

The normal distribution is a very deeply rooted phenomenon of nature, which can be found even in nonliving systems. For example, the frequency distribution of the velocity of mercury atoms at 100°C shows a perfect normal distribution (see Ulich and Jost, 1963). It is interesting that a conversion of the frequency distribution of the velocities to cumulative frequency would yield a slope similar to the slope of a dose-response under ideal conditions (toxicokinetic or toxicodynamic steady state) particularly if presented on a logarithmic scale.

Hayes (1991) was fully aware of the potential complexity of a realistic interpretation of a biochemical lesion when arising as a result of a simple effect (Michaelis-Menten) or of a complex (Hill) effect. However, he did not point out the equivalency of those statements with statements using time as an explicit function of toxicity:

$$Effect = \frac{MaximumEffect \times [S]}{K_D + [S]} \quad Effect = \frac{c \times t}{k}$$

$$\text{Effect} = \frac{MaximumEffect \times [S]^x}{K_D + [S]^x} \quad \text{Effect} = \frac{c \times t^x}{k}$$

In the simple case (Michaelis-Menten), both equations depict rectangular hyperbolas, whereas in the cooperative case (Hill), they represent nonrectangular hyperbolas. It is remarkable how much effort went into avoiding time as an explicit function of effects and to express this functionality through substrate concentration and a rate constant (in which time as a variable is concealed) instead of measuring actual time-dependence of effects.

1.3.2 Measurement of Cumulative Effects

1.3.2.1 Early Methods

Compounds differ in their tendency to cause cumulative effects when administered repeatedly. The need for a method to express this tendency quantitatively is so obvious that several independent attempts have been made to improve on the old method of stating what fraction of a one-dose LD 50 could be tolerated daily. Apparently the first method that involved statistical concepts was that of Lim *et al*. (1961). It consisted of an increasing dose, two-group (ID_2) schedule according to which each drug was started at 9% of the acute LD 50 rate and was increased by a factor of 1.5 every 4 days until the animals died (usually in 3–4 weeks). The starting point and the rate and interval of increasing the dosage were chosen in such a way that most tests could be finished in 24 ± 4 days. The chronic LD 50 was expressed as a percentage of the acute LD 50 for the same compound. Lim *et al*. (1961) showed that their method distinguished successfully (and rapidly) between drugs that were known to be cumulative in effect (chronic LD 50, 5–71%), those that show no important cumulation or tolerance (chronic LD 50, 91–102%), and those that induce tolerance (chronic LD 50, 137–467%). The use of increasing doses (which corresponds to few if any situations in the medical use of drugs or in the exposure of anyone to xenobiotics) apparently was sufficient to discourage adoption of this method.

The work of Lim *et al*. (1961) affords the opportunity to illustrate the power of the Rozman and Doull theory to understand the early search for empirical explanation/exploration of the relationship between dose and chronicity, the latter being a surrogate word for time as the second independent variable of toxicity. It is important to emphasize that according to Fig. 1.1 the first and decisive question to be answered is: Where does the rate-determining (limiting) step(s) come from? Dynamics or kinetics? This can be answered unequivocally if the dynamic (effect) and kinetic(compound) half lives are known because whichever is slower will provide that (those) step(s). The kinetic half life of HpCDD is in the order of 350 days and therefore according to Rozman *et al*. (2005) this provides the rate-determining step for this compound. Thus, the differential equation on page three applies to this compound. Because of essentially linear accumulation of HpCDD, the acute LD 50 of this compound would have been reached before day 20 after Lim *et al*. (1961)'s dosing regimen. Clearly, he had some compounds with relatively long half-lives (resepine, emetine, digitoxin, bromide) and therefore accumulation of compound. However, none had near the very long half-life of HpCDD. His second category is easiest to understand. If the acute LD 50 is much less than the (cumulative) (sub) chronic dose then it amounts to giving single daily dose rates with either nearly complete elimination (kinetic) or recovery (dynamic) every day. In that case the last daily dose rate would have been about 90% of the acute LD 50. Clearly, he had in this category compounds of very short kinetic half lives (acetylsalicylic acid, nalorphine) and therefore no accumulation of compound and for lack of a long dynamic half-life also no accumulation of effect. His last category, depicted as developing tolerance, consists mostly of two types of drugs: receptor-reactive agents (morphine, phenmetraxine, 1-phenylephrine) and enzyme inducers (prochlorperazine, chlorpromazine, phenobarbital, pentobarbital). Tolerance – a form of adaptation – is usually associated with legal or illicit drugs. Development of tolerance to the first type of drugs is clearly due to receptor up-regulation, which is a dynamic phenomenon. Since all the above drugs have short kinetic half-lives it is obvious that the rate-determining step (responsible for the up-regulated receptor steady state) originates from the slow recovery half-life of the receptor up-regulation. The four enzyme inducers all have kinetic half-lives in excess of 1 day, implying that it takes 4+ days to reach kinetic steady state and as many to return to base line. Increased synthesis/degradation of enzyme protein is faster than that, implying that the rate-determining step originates in the kinetics of these drugs, the end result of which is enzyme induction resulting in increased clearance of these drugs. Thus, understanding tolerance in light of the Rozman-Doull theory allows to understand tolerance as originating either in the dynamics or the kinetics of the underlying adaptation.

The first statistical method for measuring cumulative effect that remains in use was developed in the Soviet Union (Kagan, 1964; Kagan and Stankevic, 1964). According to this method, the result is expressed as a cumulation coefficient *(K_{cum})*.

Whereas a definition of this term (K_{cum} = LD 50_n/LD 50_1 or K_{cum} = chronic LD 50/acute LD 50) has been widely circulated in English (Kagan, 1970, 1975), a detailed explanation of the method that would permit any investigator to apply it to his or her own data has not been readily available. Such a detailed explanation did appear as Annex III in a book issued by the All-Soviet Scientific Research Institute of Hygiene and Toxicology of Pesticides, Polymers, and Plastic Materials (Hygiene and Toxicology of Pesticides, Polymers, and Plastic Materials, 1969, hereafter); and the method is discussed in Section II, 2 of that text (pp. 25–29). This book may have escaped the attention

of some English-speaking toxicologists. Fortunately, critical parts of the report were translated (but not published) by the World Health Organization and were made available by Dr. M. Vandekar.

According to Dr. Kagan's method, the chronic LD 50 is determined by plotting (on logprobit paper) each total dosage against the percentage mortality it produced. The LD 50 is then read off the graph from the 50% mortality intercept. The total dosage for each animal is determined by multiplying the number of days it survived by its daily intake of toxicant (mg/kg/day) derived from measured food intake and from the concentration of the toxicant in the feed available to the particular group. Only one point is plotted for each day on which one or more animals die. Thus, for a group in which 10 animals receive a compound at a dosage of 1.5 mg/kg/day and in which one animal dies on day 10, two more die on day 12, and so forth, the total dosage plotted for 10% mortality is 15 mg/kg; for 30% mortality, 18 mg/kg; and so forth. The results for each group of animals are plotted separately, providing a single LD 50. Each LD 50 is divided by the same one-dose LD 50 that has been determined in the usual way in a separate experiment; the quotient is the coefficient of cumulation (K_{cum}) for the particular group and, therefore, for the particular dietary level of compound. No way of statistically combining the results from different groups has been suggested. Instead, the coefficients obtained from different groups are evaluated separately in terms of their numerical relationship to the daily dosages that contributed to them.

Various methods of using these coefficients to evaluate the cumulative effects of a compound are in the literature (Kagan, 1970, 1975; Hygiene and Toxicology of Pesticides, Polymers, and Plastic Materials, 1969). According to one method, coefficients less than 1.0 signify high cumulation; those greater than 5.0 signify slight cumulation.

Kagan's method was used to determine coefficients of cumulation for warfarin and parathion, using the same raw data used earlier (Hayes, 1967b) to measure the chronicity index for those compounds as described in Section 1.2.2.3. Briefly, the methods agreed in showing that warfarin produces cumulative effects and parathion does not. However, use of the coefficient of cumulation presents practical difficulties. The exact coefficient obtained for a compound varies with the dosage chosen. The major difference between the method of Kagan and those of Boyd and Hayes is that results from only a single group of animals are considered in a single statistical maneuver. Even when several groups are studied and the different resulting coefficients are plotted to form a curve, there is no statistical integration of the results for the separate groups to generate a single coefficient. By contrast, each of the other two methods requires *(a)* the use of several dosage groups and *(b)* the statistical integration of the results from all of the groups studied. It is concluded that Kagan's method is not as precise as either of the basically similar methods proposed by Boyd or Hayes.

Kagan's method is easily understood in the context of Section 1.1.2 in combination with Fig. 1.1. He plotted the cumulative dose rates (dose) of multiple-dose-rate studies vs. effect on a logprobit paper, which is theoretically legitimate, because the dose is always the sum of all dose rates. Accumulation of effect is either the result of accumulation of toxicant or accumulation of injury, whichever has the longer half-life will dominate the dynamics of injury. If there is no recovery from injury or no elimination of toxicant, then there will be linear accumulation of injury or toxicant according to a triangular geometry after multiple dose rates or according to a rectangular geometry after a single loading dose rate followed by maintenance dose rates. Thus the lowest theoretical value for $K_{cum} = 0.5$. Indeed a value of less than 1.0 was considered an indication of accumulation, whereas one greater than 5 was considered an indication of slight accumulation only (Hayes, 1991). The explanation for warfarin causing and parathion not causing a cumulative type of effect is a direct consequence of an appropriate understanding of the role of time in toxicology. The kinetic half-lives of both warfarin and parathion are shorter than their dynamic half lives. Therefore, the dynamic half-lives will dominate the actions of both of these compounds. The dynamic half-life of warfarin is about 1 day (Nagashima *et al*., 1969). Thus, 90 and 99% of dynamic steady state will be reached after 3.32 and 6.64 dynamic half-lives of continuous exposure, respectively, after which accumulation of effect will occur according to $c \times t = k$ with only a 25% difference between C_{max} and C_{min} assuming two bouts of feeding per day. The recovery half-life of parathion is less than 12 h estimated based on data available for soman (Rozman, 2000a). Thus, steady state for parathion will be reached more rapidly but at an average level less than half of that for warfarin with larger fluctuations between C_{max} and C_{min}. Depending on the dose selection, the cumulative effect of warfarin may be above the 90-day $c \times t$ threshold whereas that of parathion may be below it.

1.3.2.2 C/A Index

The next statistical method to be introduced was that of Boyd *et al*. (1966), who suggested that the comparison be made at the LD 50 levels for both the acute and the subacute tests. Specifically, he proposed that a one-tenth life span (0.1L) chronic/acute LD 50 (0.1L) *index* [C/A LD 50 (0.1L) index] be calculated by expressing the multiple-dose LD 50 as a percentage of the acute LD 50. (Both kinds of LD 50 values involved stomach tube administration, but the acute dose was given to nonfasted animals.) The C/A index for sodium chloride was found to be 72, indicating that 100 daily doses of table salt each at a rate 72% of the acute LD 50 would kill half of a very large population of rats.

The C/A index, unlike Kagan's cumulative-dose-rate (= dose) vs. single-dose-rate coefficient, relates the single dose rate of a multiple-dose-rate study to a single-dose-rate experiment (chronic LD 50/ acute LD 50) and expresses this

ratio as a percentage. Hayes (1991) used the reciprocal of that ratio multiplied by 100 as an index to characterize the cumulative nature of an effect. He was also aware of the difference between accumulation of compound and accumulation of injury, and that his index did not distinguish between the two. Comparing multiple dose rates (daily doses or dosages) with a single dose rate (single dose) is problematic because it confuses the issue that no matter what happens to the toxicant, the organism was still exposed to the sum of the dose rates, which is the dose, just as the organism is exposed to the total dose after administration of a single dose rate. The lack of conceptualization of subsequent events which occur on different timescales led to a great deal of confusion in toxicology. As eminent a scholar as Druckrey held the erroneous view about reinforcing effects of small dose rates, because he compared single daily doses (dose rates) instead of doses (sum of dose rates) in diethylnitrosamine-induced cancer. The dose rate (daily dose) causing 100% cancer in about 60 days was 200 times higher than that causing cancer in about 900 days. In fact, the doses were only different by a factor of 12, which represents the specific time response (Fig. 1.2) at constant dose rate (steady state).

Both the C/A index and the index of Hayes are useful as indicators of chronicity. Nevertheless, their lack of distinction between the different timescales involved conveys incomplete and often erroneous messages. High chronicity index for mirex is indicative of strong cumulative toxicity. However, the more important information that the cumulative AUCs after a single dose rate (1-dose LD 50) of mirex are virtually the same as after 90 fractionated dose rates (90-dose LD 50), indicating a slight (40%) adaptation to mirex, is not revealed. Similarly missing is the important information that the toxicity of mirex is largely dominated by its kinetic half-life (ATSDR, 1995a) of about 350 days

TABLE 1.2 Absolute and Relative, Acute and Subacute Oral Toxicity of Certain Pesticides and Drugs[a]

Compound	Species	Sex	1-Dose LD 50 (mg/kg)	90-Dose LD 50 (mg/kg/day)	Chronicity index
Mirex	Rat	F	365	6.0	60.8
Warfarin	Rat	M	1.6	0.077	20.8
Metepa	Rat	M	136	7.5	18.1
Dieldrin	Rat	M	102	8.2	12.8
Atropine	Rabbit	M	588[b]	78[b]	7.5
Apholate	Rat	M	98	17	5.8
Paraquat	Rat	F	110	20.5	5.4
DDT	Rat	M	250	46.0	5.4
Benzylpenicillin	Rat	M	6700[c]	4140[c]	1.6
Sodium chloride	Rat	M	3750[d]	2690[e]	1.4
Caffeine	Rat	F	192[f]	150[g]	1.3
Parathion	Rat	F	3.6	3.1	1.16
				3.5	1.03
Azinphosmethyl	Rat	F	11.0	10.5	1.05
EPN	Rat	F	7.7	12.0	0.64
Dichlorvos	Rat	F	56	>70	>0.08
Potassium cyanide	Rat	M	10	<250[h]	>0.04

[a]From Hayes (1967b) or later U.S. Public Health Service data, except as noted, by permission of Academic Press. The compounds are listed in approximate order by decreasing chronicity index.
[b]Boyd and Boyd (1962) (100-intramuscular-dose test).
[c]Boyd and Selby (1962 (100-dose test).
[d]Boyd and Shanas (1963).
[e]Boyd et al. (1966) (100-dose test).
[f]Boyd (1959).
[g]Boyd et al. (1965) (100-dose test).
[h]No mortality occurred at 250 mg/kg/day, the highest dosage administered.

(in rats). The toxicity of warfarin represents the opposite end of the spectrum because it is determined by the dynamics of the effect as discussed earlier. A chronicity index of 20.8 is indicative of cumulative toxicity, but it is misleading in that it suggests the toxicity is less cumulative than that of mirex. In fact, considering that no rat died related to administration of warfarin in the 90-dose-rate study after day 25 shows that the chronic dose (sum of dose rates) was a mere 1.2 higher than the acute dose (one dose rate) indicating nearly perfect $c \times t = k$, implying chronicity similar to mirex.

1.3.2.3 Chronicity Index

The *chronicity factor* introduced independently by Hayes (1967b), is expressed as a quotient rather than a percentage. However, this factor is really an index and ought to be designated as such in the future. Excluding differences in the procedures for measuring the LD 50 values, the chronicity index for a compound is the reciprocal of its C/A LD 50 (0.1L) index expressed as a fraction instead of as a percentage. That is,

$$\text{Chronicity index} = \frac{100}{\text{C/A LD50}(0.1L)index}$$

For example, the C/A LD 50 (0.1L) index for sodium chloride (71.7) would correspond to a chronicity index of 1.395.

Because each chronicity index is a ratio, these indices may be used to compare the tendency of different compounds to have cumulative effects without reference to their absolute toxicities. This index is determined on the basis of an observed effect. No distinction is made between effects that depend in part on cumulation of the toxicant (e.g., lead) and those that do not (e.g., alcohol).

The chronicity index for each compound is obtained by dividing its 1-dose LD 50 (expressed as milligrams per kilogram) by its 90-dose LD 50 (expressed as milligrams per kilogram per day). The resulting number is large (2.0 or more) for compounds that are relatively cumulative in their effects and small (less than 2.0) for compounds that show little cumulative effect. The index of 2.0 is recognized as an arbitrary dividing point, but it appears supported by the limited data available and is also plausible on theoretical grounds. In any event, if a compound were absolutely cumulative (in the sense that 1/90 of the 1-dose LD 50 was exactly the 90-dose LD 50), the chronicity index would be 90. A chronicity index of 1.0 associated with oral intake indicates that daily ingestion of the 1-dose LD 50 mixed into the regular diet leads to death of half of a very large population so exposed for 90 days, which is very difficult to verify experimentally, but sodium chloride gets as close to it as experimentally possible.

Table 1.2 summarizes the 1-dose and 90-dose LD 50 values and also the chronicity indices for warfarin and several other compounds. The marked cumulative effect of warfarin and the chemosterilants; the small magnitude of such an effect of table salt, caffeine, and some organic phosphorus compounds; and the essential lack of cumulative effect of potassium cyanide are recorded. The 90-dose LD 50 of warfarin was only about 1/20 of the 1-dose LD 50, indicating a chronicity index of about 20 for that compound. It required daily ingestion of approximately a 1-dose LD 50 of several organic phosphorus insecticides to kill half of the test animals in 90 days, indicating a chronicity index of approximately 1 in each instance. Rats tolerated daily 25 1-dose LD 50s of potassium cyanide mixed with their regular food with no mortality, indicating a chronicity factor of less than 0.04. This tolerance for organic phosphorus compounds and cyanide undoubtedly indicates the ability of the body, and especially the liver, to detoxify moderate dosages of these materials provided there is time in which to accomplish the task. The chronicity index permits comparison of the effects of different classes of compounds of the same class. Whether these smaller intraclass distinctions are really significant or whether they are outweighed by differences caused by species or other factors must be determined by future experience. It is certainly to be hoped that increasing use will be made of 90-dose LD 50 and ED 50 values and of the chronicity index in order that the study of long-term toxicity may be made more quantitative.

The chronicity index is a measure of cumulative effects. A concentration index has been proposed as a measure of cumulative storage. The effect of a compound cannot be less than that determined by its storage in the body, especially its presence (storage) in sensitive tissues. In this sense, a compound that has a high concentration index will tend to have a high chronicity index. However, some compounds are highly cumulative in their effects even though they show a minimal tendency to storage. Thus, the two indices do not vary in a parallel fashion.

It is generally agreed that what has been called biological magnification is the basis for the injury caused by DDT and a few other compounds to certain large, predatory forms of wildlife. Biological magnification occurs in situations in which a compound shows a high concentration index in each successive species in a food chain.

This section demonstrates that Hayes (1991) was fully aware of the importance of time in the manifestation of toxicity without generalizing time as an equivalent and fully quantifiable variable of toxicity along with the dose. Perhaps for this reason, he made no reference to measuring time accurately in toxicological experiments. There are additional issues to be considered when viewing time as a variable of toxicity: the timescale on which the effect is occurring and the frequency of observation, which are related to each other as well as to the half-lives of compound or effect and exposure frequency. A clear distinction must also be made whether dose-time or effect-time relationships are being considered because the former requires

the study to be conducted at constant effect, whereas the latter necessitates an experiment at constant dose (steady state). Routine daily observation of experimental animals in chronic experiments arises out of practicality and is (e.g., cancer studies) without scientific rationale. In fact, in two-year or longer lasting cancer studies weekly observation would yield satisfactory time resolution of the cancer latency period (but not for harvesting tissues). However, the daily observation of animals in acute experiments often provides worthless information on that timescale if all the animals die within 2–3 days or even sooner. Automated cameras could provide hourly or continuous monitoring, which would result in the necessary accuracy for quantitative time relationships. Toxicant-induced reduced sea urchin sperm motility occurring on a timescale of hours is only meaningfully measured on a timescale of minutes and nobody in his right mind would want to study pungency on a timescale of minutes when it requires time resolution on a scale of seconds. This all sounds simple and straightforward, yet cookbook-type toxicology is devoid of these simple considerations. The timescale on which an effect is occurring is important for several reasons. The length of the observation period for any experiment should be the time required by a LOAEL to cause the effect. The relationship between the time to an effect and the dynamic or kinetic half-life of an effect are other critical variables which often confuse toxicological experiments, because the respective rate-determining time ratios will introduce different ratios of intoxication/recovery as discussed in Section 1.3.1.1 with dieldrin and toxaphene as examples. It must be recognized that our understanding of time is perhaps more limited than mankind's understanding of matter was during the era of Paracelsus and yet toxicology is one of the few fields that can open the gate to the structure of time.

1.3.3 Time Relationships

The report of essentially every toxicity test should include information on the time relationships of the effects observed. It is important whether a single dose produces its effect soon after dosing or only after hours or days and whether the effect is brief or prolonged. From a practical standpoint, it is important to know whether a patient, who is mildly sick an hour after overexposure to a toxicant, is really "over the worst of it" or likely to slip at any moment into a critical condition. From a theoretical standpoint, rapid onset of illness following dosage of experimental animals at or below the LD 50 level suggests that the toxicant is absorbed rapidly and acts directly. Rapid recovery following dosing at a substantial rate suggests that the toxicant is excreted or detoxified rapidly. On the other hand, slow onset of illness following dosing at almost any level suggests that the toxicant is absorbed slowly or must be metabolized before it can act. Prolonged illness following dosage at or below the LD 50 level usually suggests that detoxification and excretion of the toxicant are inefficient, but sometimes means that the toxicant produces some anatomical or biochemical lesion that recovers slowly or not at all.

Time relationships in toxicity often can be expressed best by recording the range and mean of time required to produce an observed effect. However, a more elaborate statistical treatment sometimes is indicated. An ET 50 or LT 50 gives a more precise estimate of time to be anticipated in repeated tests than can be expressed by a simple average. The log-time-logdose curve (Section 1.3.3.2) has also considerable theoretical interest and, in some instances, may be used to predict the proper dosages to be used in long-term studies. In this paragraph Hayes truly anticipated developments in theoretical toxicology that occurred after his death.

1.3.3.1 ET 50 or LT 50

An ET 50 is a statistical estimate of the interval or time from dosage to a specific all-or-none response of 50% of the organisms in a very large population subjected to a toxicant under specified conditions. As used here, an all-or-none effect may be a specified level of a quantitative response: for example, time of appearance of the first tumor or time at which the systolic blood pressure reaches 150 mm Hg. An LT 50 is a special case of an ET 50 in which the effect reported is death. ET 50 and LT 50 values are determined by relating the cumulative percentage effect. In practice, the calculation (Bliss, 1937) or graphic solution (Litchfield, 1949) is carried out in a manner essentially identical to those used for ED 50 and LD 50 values.

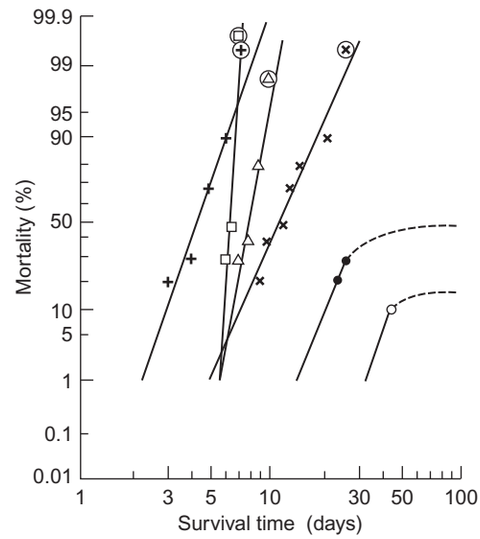

FIGURE 1.10 LT 50 curves for male Sherman strain white rats administered repeated dosages of warfarin. Dosages (mg/kg/day) were as follows: (O) 0.04; (●) 0.08; (×) 0.16; (△) 0.32; (×) 0.64; (+) 1.28. Points adjust according to the method of Litchfield and Wilcoxon (1949) are distinguished by a superimposed O.

There is one striking difference in the form of these statistics for dosage and time. In considering dosage *per se,* the time of response is ignored completely. A series of tests involving several dosage levels of a compound results in a single dosage-response curve. On the other hand, in considering time of response, dosage cannot be ignored, and a series of tests involving several dosage levels results in a series of separate curves of different slope. A sufficiently low dosage of any compound will generate a curve coinciding with the baseline, indicating that no animals were affected. The critical range will generate a series of curves such that both the slope and the magnitude of the ET 50 are inversely proportional to dosage. In general, progressive increase of the dosage beyond that necessary to affect all animals will cause progressively less and less change in the slope and position of the ET 50 curves. However, in some instances, progressive increase in dosage beyond that necessary to kill all animals will cause a relatively sudden shift of the very-high-dosage ET 50 curves to the left accompanied by an unpredictable change in their slopes. Such a change indicates that a different mode of action has begun. Any dosage above that necessary to kill all organisms in a population is a *supralethal* dosage, but the term is used most often in connection with dosages that involve some difference in mode of action. Examples may be found most commonly in the toxicology of compounds of which the ordinary effects are delayed. Such compounds are discussed further in Section 1.3.3.2.

Except for the phenomenon of changed mode of action, the points discussed in the last paragraph are illustrated by Fig. 1.10 which shows LT 50 curves resulting from different dosage levels of warfarin administered in connection with a 90-dose LD 50 study. (Similar LT 50 curves were obtained in connection with a 1-dose LD 50 study.) It may be seen from Fig. 1.10 that, in practice, the progression of curves from right to left is not always completely orderly. The curves at the right tend to be horizontal or incomplete (indicated by dashed lines) because only a portion of the animals in these groups die. The curves at the left tend to approach the vertical, but there is some irregularity, caused no doubt by individual differences and the fact that only a limited number of animals are used in each group. (The data on which Fig. 1.10 was based were used in a different form in connection with the corresponding dosage levels in Fig. 1.12 (see Section 1.3.3.2). A comparison of the two figures shows the value of the two kinds of graphs for illustrating different aspects of the same results.)

It is amazing how clearly Hayes (1991) saw the problem of time being ignored when "considering dosage *per se.*" Section 1.1.4 deals with this problem by defining mathematically that the toxic action of chemicals consists of a specific effect at constant dose plus a specific effect at constant time. He was also keenly aware of the fact that the time response curves do not progress in an entirely orderly manner at supralethal (supraeffective) doses because sometimes the mode of action changes. However, if that is not the case and the mode of action remains the same then there is a highly orderly decline in the slope of the time response regarding mortality (Rozman, 1999) or other effects (Gardner *et al*., 1977).

Figure 1.10 is plotted on a double logarithmic scale which is not as sensitive to deviations from a straight line as is the single logarithmic plot applied by Gardner *et al*. (1977) and Rozman (2000a). Single logarithmic plots of time-responses yield S-shaped curves similar to dose-responses in log(dose) vs. effect plots, again representing the inverse function of the theoretical plot represented by log(effect) vs. time (arithmetic).

1.3.3.2 Logtime-Logdosage Curve

Compounds may show one or more of the following interrelations between dosage and time to response, regardless of whether there are one or more doses: (a) a uniform delay between the first dose and the response; (b) a prolongation of the interval that is inversely related to dosage; and (c) a complete absence of detectable effect at low dosages and, therefore, an interval that exceeds the period of observation (which may be the lifetime of the subjects).

Delayed Toxicity As is well known, there is a lag in the appearance of a detectable effect of some compounds. In other words, there is an inherent delay in their action, which is not accounted for by the time necessary for their absorption

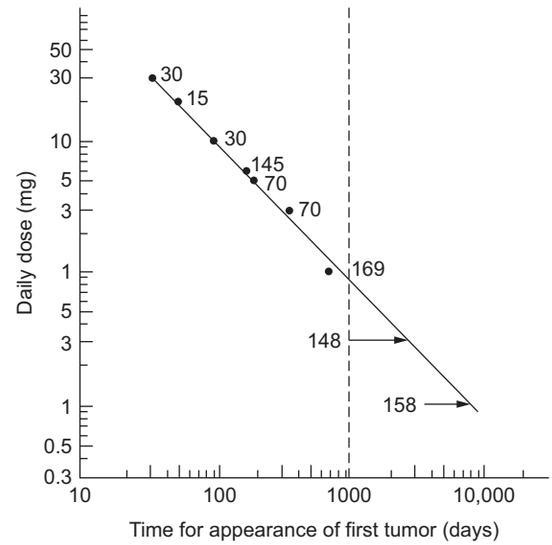

FIGURE 1.11 Response of rats to graded daily of 4-dimethylaminoazobenzene (4-DAB) administered orally. No liver tumor was obtained with the two lower doses employed, indicated by arrows. The number associated with each point or arrow refers to the number of animals tested at that dosage. The dotted line represents a life span of 2.74 years. From data of Druckrey (1959).

and distribution to the target organ. The inherent delay is not fully overcome by substituting larger doses or by using intravenous injection. Examples are offered by (a) carcinogens, for which an induction period apparently always is required, (b) certain organic phosphorus compounds that produce paralysis in humans or chickens, but only following a delay of about 10 days, and (c) the coumarin-derived anticoagulants, which inhibit prothrombin formation but have little or no initial effect unless the dosage is massive and a different toxic mechanism is involved. Some other compounds, notably alkylating agents (Hayes, 1964), produce delayed effects but generally produce some illness promptly after a dose at or even below the LD 50 level.

A compound cannot produce a delayed effect unless it or its metabolites or a direct or indirect pharmacological action persists until the clinical effect appears. This persistence of a compound or its action is the essence of which cumulative effects are made. On the contrary, the coumarin-derived anticoagulants exhibit the delay but do not produce a truly chronic disease. Whereas the biochemical basis for the delayed action of coumarin-related anticoagulants is understood clearly, the reasons for the delays associated with other agents are obscure, probably because we do not yet know the biochemical lesions involved. It is conventional to explain the delay associated with carcinogenesis as the result of a "multistage process" but this is more a phrase than an explanation.

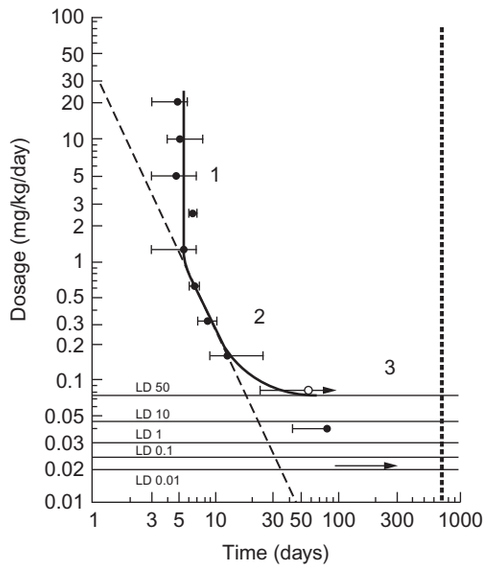

FIGURE 1.12 Relationship between dosage of warfarin and time of death in rats. Short horizontal line indicates the time of death of the first animal in a group to die, and a solid point indicates the geometric mean time to death for a group in which all the animals died. In those groups in which some animals did not die, their survival is indicated by an arrow, and the best estimated geometric time to death is indicated by an open circle. The tip of each arrowhead indicates the end of the dosage period. Because it could not be assumed without produce that the survivors would live a normal life span, it was empirically assumed for purposes of calculation that the survivors died on the last day of the test. Thus, the true position of these estimated values always lies to the right of the open circle. Note that no rats died when dosed for 300 days at a rate of 0.02 mg/kg/day. The graph also shows the 90-dose LD 50 and some other dosage response values for 90 doses of warfarin. Dotted vertical line represents average life span. Slightly modified from Hayes (1967b), by permission of Academic Press.

The term "delayed toxicity" ought to be restricted to delay in onset of clinical effects following the absorption of an adequate dosage. The term ought not to be used to refer to (a) a delay in onset that depends on the time necessary for the accumulation of an adequate total dosage from relatively small repeated exposures or (b) the progression of disease, including any that is the result of scarring or some other morphological or biochemical effect that is an inherent part of the toxic injury.

Although the necessary delay in the onset of the effect of some compounds is well known, it has not been customary to represent it graphically. By contrast, curves relating the increasing interval from the first and sometimes only dose until the appearance of a selected effect were used at least as early as 1937 and were based on data published as early as 1908 (Clark, 1937). Clark showed that, for a certain range of dosages characteristic of each test system, there often is a linear relationship when the logarithm of time to response is plotted against the logarithm of dosage producing the response. Both the graphic and the mathematical features of the relationship were thoroughly investigated by Bliss (1940). Figure 1.11 indicates the form of a typical curve. Recognition of the relationship apparently has not been general, with the result that it has been rediscovered from time to time.

It is a general principle of toxicology that any compound may be tolerated without injury provided the dosage is sufficiently small. It has not been customary to represent this relationship graphically. As discussed later, such representation is desirable, for it reveals what may be basic differences in the behavior of different compounds.

Shape of the Complete Logtime-Logdose Curve

Summarizing the last several paragraphs, it is evident that a complete logtime-logdose curve would have three segments:

1. The first segment represents the minimal time necessary to produce an effect even with dosages larger than the minimal one required.
2. The second segment represents the increasing times necessary to produce an effect with successively smaller dosages.
3. The third segment indicates a dosage a little below which the effect is not produced, no matter how long dosing may be continued.

Such a curve based on a study of warfarin is shown in Fig. 1.12. The three segments are well shown. The second

segment was established by dosages of 0.16, 0.32, 0.64, and 1.28 mg/kg/day, and in each instance the time necessary for half of the animals to die was 10 days or less. (The points in Fig. 1.12 represent geometric means as explained in the legend. LD 50 values could have been used and would have given essentially identical results. The choice was based on convenience, especially in connection with groups in which only a few animals died.)

As may be seen, the third segment in Fig. 1.12 has been drawn out horizontally at the level of the 90-dose LD 50 value for warfarin as determined by the original, detailed form of the 90-dose curve in Fig. 1.6. The corresponding LD 10, LD 1, LD 0.1, and LD 0.01 have been indicated also. Because the lowest dosage tested (0.02 mg/kg/day) lies between the values calculated to be the LD 0.1 and LD 0.01 levels, there is little wonder no effect was observed among a group of only 10 animals.

It appears that a few compounds (e.g., warfarin in Fig. 1.12) exhibit all three segments of the theoretical curve, some compounds (e.g., 4-DAB in Fig. 1.11) exhibit the first and second segments only, and most compounds exhibit the second and third segments only. Perhaps some compounds exhibit the second segment only, but no illustration is available. It is impossible to make a more exact statement at this stage because there has been so little study of comparative, quantitative toxicology. In fact, it is not established that all compounds exhibit a typical second segment of the theoretical curve, although this appears likely. Curved second segments shown by some authors (Clark, 1937) may, in fact, represent a transition between second and third segments.

The presence of a delayed reaction following large dosages does not exclude the possibility that small dosages of the same compound may be tolerated. Figure 1.12 offers an illustration of this kind of tolerance in rats fed warfarin. Another example is offered by the work of Siegel *et al.* (1965), who showed that a mixture of tricresyl and other triaryl phosphates, which produce paralysis of chickens and rabbits after only 20 days of high-level exposure, was tolerated by both species for as long as 90 days when given at lower dosage levels. Of course, it is easy to demonstrate tolerance for small dosages of most compounds, albeit they do not elicit a significant latent period when absorbed at high dosage levels.

In all instances studied so far, carcinogenesis is associated with lack of a third segment in the logtime-logdose curve. In connection with toxicity generally, lack of this segment is exceptional. The presence of the third segment strongly implies the existence of a threshold at a level only a little less than the level of the segment itself. It is not certain how the absence of a third segment ought to be interpreted, but there is no evidence to exclude the possibility that a threshold exists here also at a dosage level just below that required to produce the smallest statistically significant increase in the incidence of tumors above control levels in animals that survive as long as any of their species. Regardless of the logtime-logdosage-response, the existence of a threshold should be demonstrated biochemically, as discussed in Section 1.3.7.4.

Substances	First used by	$c \times t$
Ethylbromoacetate	France	3000 and less
Chloroacetone	France	3000
Xylylbromide	Germany	6000
Chlorine	Germany	7500
Perchlormethyl mercaptan	France	3000 and less
Hydrocyanic acid*	France	1000
Phosgene	France	450
Methylchloroformate	Germany	500

**The value of ($c \times t$) for hydrogen cyanide depends on its concentration. The value given refers to the concentration of 0.5% obtainable in the field. The values are much higher with smaller concentrations. (Reprinted from Haber (1924), by permission of Springer-Verlag.)*

Use of the Logtime-Logdosage Curve for Prediction

Aside from its basic interest for toxicology, the logtime-logdosage curve may be used in connection with a brief test to predict appropriate dosage to use in long-term studies. Reference to Fig. 1.11 shows that only about 90 days of testing of 4-DAB in rats at dosages of 10, 18, and 32 mg/rat/day would have been sufficient to predict that a dosage of about 0.9 mg/rat/day produces an effect within the lifetime of that species. On the contrary, use of the same technique in connection with warfarin (Fig. 1.12) predicted a limiting value which, however, did not correspond closely with the value actually observed in a long-term experiment. By extrapolation of the second segment of the curve (as shown in part by the dashed line in Fig. 1.12), one would predict that a dosage of approximately 0.002 mg/kg/day would kill half of a sufficiently large group of similar animals within 90 days. This prediction for warfarin is seriously inaccurate when compared with the 90-dose LD 50 of 0.077 mg/kg/day based on all the dosage levels tested, including a dosage of 0.020 mg/kg/day, which was tolerated for 300 days without any mortality.

The fact that the value predicted may be only limiting and may not correspond closely with observed long-term toxicity does not make the test useless. The test does have the advantage of relative brevity. It is better to know a limiting value than to have no valid guide for choosing dosages for long-term study.

The logtime-logdosage curve also may be used in the bioassay of bacterial and other toxins. What may have been

the first effort along this line (Boroff and Fitzgerald, 1958) confirmed the linear log-log relationship demonstrated earlier for other substances and showed that, by the intravenous injection of relatively large doses, a test could be completed in less than two hours instead of the four days required for the conventional test for toxins. It was shown later that, by using dilutions that had been tested in the conventional way, it was possible to construct a standard curve relating the logarithm of the mean survival time in minutes to the logarithm of concentration or logarithm of the number of lethal doses per unit volume, thus providing a prompt measurement expressed in the desired unit (Boroff and Fleck, 1966).

Haber's Rule Apparently the only statement Haber made of what has come to be called his rule is contained in a footnote to the last of a series of five lectures this Nobel Prize-winning chemist made in the years 1920–1923 (Haber, 1924). This particular lecture concerned the history of gas warfare, and all the toxicological considerations were in this context. This means, among other things, that only brief exposures were considered. At that time, no chemical was known that would not drift away or be diluted to a harmless concentration soon after its release. The complete footnote may be translated as follows:

A simple and practical measure for toxicity can be obtained that suffices for all practical purposes. For each war gas, the amount (c) present in one cubic meter of air is expressed in milligrams and multiplied by the time (t) in minutes necessary for the experimental animal inhaling this air to obtain a lethal effect. The smaller this product $(c \times t)$ is, the greater is the toxicity of the war gas. A few values obtained during the war are given in the table. More detailed information can be found in the medical literature. The values were all obtained by using cats as experimental animals. The chemicals are listed according to the order of their introduction as war gases.

It may be noted that the footnote implies but does not state what is now called Haber's rule for equitoxic doses, especially fatal doses, namely,

$$c \times t = k$$

where c is concentration, t is time, and k is a constant characteristic of a particular compound.

Actually, the concept was not original with Haber. Apparently it was stated first by Warren (1900) in connection with his studies of the effect of different concentrations of sodium chloride on *Daphnia magna.* Warren stated the relationship as

$$T(X - 8) = \text{constant}$$

where T is the time of killing and X is the strength of salt solution. The value 8 was an observed constant concentration below which the relationship did not hold and survival of the animals was influenced little or not at all by the salt.

TABLE 1.3 Dosage Relationship for the Second Segment of the Curve in Fig. 1.12

Daily dosage (mg/kg)	Total dosage (mg/kg)
1.28	7.17
0.64	4.28
0.32	2.69
0.16	2.03

Thus, Warren recognized that the relationship was true only within certain limits of time and concentration. It is clear from his note on hydrocyanic acid that Haber, too, was fully aware of the limitation of the constant relationship.

Bliss (1940) reviewed some earlier papers on the subject and presented an elaborate mathematical analysis of the relationship between exposure time and concentration.

Restatement of Haber's Rule Further study has emphasized the limited applicability of the rule. Recognition of this limitation has led to a restatement of the relationship as where

$$\frac{[(C \cdot V_m) - D_e] \cdot t \cdot R}{w} = D$$

D = dosage (mg/kg) received during time t
C = concentration of toxicant (mg/m^3)
V_m = minute volume rate of respiration (m^3/min)
D_e = detoxification rate (mg/mm)
t = time (min) of exposure
w = body weight (kg)
R = retention coefficient expressed as a decimal fraction

This equation shows that a sufficiently high rate of detoxification would negate prolonged exposure to a sufficiently low concentration. It thus expresses quantitatively the limitation on the rule when applied to easily detoxified materials like hydrocyanic acid. It will be seen that in this equation dosage (D) is not necessarily a constant for all combinations of concentration and time that produce the same effect, because the detoxification rate and perhaps the retention coefficient may vary with dosage.

David *et al.* (1981) evaluated the role of time and concentration on carbon tetrachloride toxicity in rats. Using hepatotoxicity as a marker and varying concentrations and time products as exposure, these authors concluded that the severity of liver lesions was more influenced by the concentration of carbon tetrachloride in the inhaled air than by the product of concentration and time. The limiting variable was the length of time required for tissues to acquire critical concentrations of the toxin.

Relation of Haber's Rule to the Logtime-Logdosage Curve If one considers the relationship $c \times t = k$ which constitutes Haber's rule, it is clear that it represents a special case of the second segment of the logtime-logdosage curve (when the dosage is expressed as concentration). When plotted on log-log paper, all solutions of the equation lie on a straight line passing through two points, namely,

$$c = 1 \quad t = k$$
$$c = k \quad t = 1$$

Furthermore, on the same set of coordinates, all solutions of all other equations of the same form will lie parallel to the first but pass through k_1, k_2 and so forth, instead of k. The slope of these lines is algebraically the same and is -1 on ordinary log-log paper.

Some logtime-logdosage curves based on observed data have a slope statistically indistinguishable from that determined by Haber's rule. Figure 1.11 shows an example. However, other real curves show greater or lesser slopes (Bliss, 1940; Clark, 1937; Druckrey, 1943, 1967; Scholz, 1965). Examination of Fig. 1.12 shows that the second segment has a downward inclination steeper than 45°. In other words, within the range of dosage from 1.28 to 0.16 mg/kg/day, the smaller dosages are progressively more effective than would be predicted by Haber's rule. That is, progressively less total dosage is required as shown in Table 1.3. In this instance, the reason is that warfarin does not depress appetite so that, at 1.28 and 0.64 mg/kg/day, the rats consumed relatively large total dosages of this slow-acting compound before they had time to die. On the contrary, at an intake of 0.02 mg/kg/day, no injury occurred even though a total dosage slightly over 5.0 mg/kg was taken in during a period of over 300 days. With the exception of the matter just discussed, the significance of slopes greater than or less than 1 is not clear; in fact, both have been observed for warfarin under different conditions. In any event, there is no relationship between the slope of the second segment of the curve and the occurrence of a third segment.

Discussion There is no meaningful relationship between Haber's rule and time-weighted averages for occupational exposure although they apparently have been confused. Both concepts involve time and concentration, but Haber's rule is an equation describing a principle of toxicology whereas a time-weighted average is a standard set to prevent overexposure of workers or others. As already stated, Haber's rule is a special case of the second segment of the logtime-logdosage curve. Each time-weighted average defines a level of exposure to a particular compound intended, often on the basis of extensive experience, to be tolerated without injury for a lifetime; this level of permissible exposure lies below and parallel to the third segment of the logtime-logdosage curve. The distance between the second and the third segment of the curve is a safety factor the magnitude of which will vary with the compound.

Investigating dose-time relationships requires a very accurate definition of effect because the experiments have to be conducted under isoeffective conditions. For example, it is not enough to state that the effect of interest is lung cancer. It needs to be specified whether it is time to first lung cancer or time to 50% lung cancer. Further specifications are needed regarding the severity of the effect: time to histologically identifiable cancer obviously should not be lumped with time to death caused by lung cancer. Even though these considerations may appear trivial, they are pivotal and unfortunately routinely ignored in the design and interpretation of toxicological experiments.

The log(dose) vs log(time) plot has its theoretical justification in Haber's $c \times t = k$ concept, the logarithmic form of which is

$$\log c = -\log t + \log k$$

which is the equation of a straight line (Fig. 1.11). The arithmetic form ($c \times t = k$) provides a rectangular hyperbola with the limits set by the minimum lag time of the effect and maximum life span of the species studied (Fig. 1.12). It is also important to recognize that theoretically all effects have a lag period between dosing and effect. Inhalation anesthesia has a very short lag period whereas cancer has a very long lag time called latency. The minimum lag period is a characteristic of the effect and therefore not subject to change. For example, HpCDD-induced delayed acute toxicity cannot cause lethality in less than 8 days even if supralethal doses are given to rats (Rozman, 1999). This lag period can have either kinetic/dynamic (delayed absorption or slow accumulation of compound) or dynamic reasons (delayed time to effect or slow accumulation of effect). Ingestion of acutely nontoxic dose rates of lead can cause a fulminant episode of toxicity once the reserve (storage) capacity of the organism has been exhausted because of accumulation of lead. A single high dose of diethylnitrosamine will cause cancer in 100% of the animals because of the persistence and hence slow recovery of the DNA damage. Section 1.0 deals in great detail with all other issues involving time that were raised by Hayes (1991). Even more information may be found on the role of time in risk assessment (Rozman, 2000a) and the use of the $c \times t$ concept in the context of establishing occupational exposure levels. Thus, we do not share Hayes (1991) view that there is no meaningful relationship between Haber's rule and time weighted averages in occupational exposure. The conditions are clearly outlined in Section 1.1, which define the conditions for Haber's rule of inhalation toxicology to become a fundamental law of toxicology. There are no exceptions to this law that we have found and therefore we suggest that any experiment that appears not to obey this law is incompletely designed or controlled. However, for Haber's rule to become manifest requires continuous exposure. Occupational

exposure is discontinuous (8 h/day, 5 days/week for 45 years), which allows for recovery or elimination 16 h between workdays and 66 h on weekends. Therefore, there is a deviation from the $c \times t = k$ or $c \times t = k \times W$ concept in the form $c \times t^x = k$ for isoeffective or $c \times t^x = k \times W$ for isodosic or isotemporal exposures in an occupational setting which requires the transformation of $c \times t^x$ back to $c \times t$ in order to establish meaningful exposure limits. This issue has been discussed and clarified by Doull and Rozman (2000).

1.3.4 Problem of Measuring Effect of Dispersed Toxicants

Although it is desirable to express dosage in terms of weight of toxicant per weight of organism, this is difficult if the dose is absorbed from air by the lungs or trachea of land animals or from water by the gills of aquatic animals. Under these circumstances, it may be convenient and even necessary to consider toxicity in terms of concentration of the toxicant in the medium. If there is continuous exposure to constant concentrations, the data are expressed in the form of EC 50 values as explained later. If time is also a variable, the time results may be presented in terms of a logtime-logconcentration curve as discussed in connection with Haber's rule.

When possible, dosage in terms of milligrams per kilogram should be calculated from the concentration of toxicant, the respiratory volume, and the proportion of toxicant retained. The result of this calculation offers one important way of comparing toxicity by the respiratory route with that by other routes. Another approach is to measure plasma or other tissue levels of toxicant following exposure by different routes (Section 1.3.6).

1.3.4.1 EC 50 and LC 50

An EC 50 is a statistical estimate of the concentration of a toxicant in the ambient medium necessary to produce a particular effect in 50% of a very large population under specified conditions. An LC 50 is a special case of the EC 50 in which the effect recorded is death. EC 50 and LC 50 values are determined similarly to ED and LD 50 values, that is, probits representing the percentages of animals showing a response in a series of tests are related to the logarithms of the concentrations that produce the responses. EC 1 and EC 99 values may be determined and confidence limits of the various estimates may be calculated. ET 50 and LT 50 values may be calculated on the basis of concentration instead of dosage.

1.3.5 Measurement of Graded Responses

What has been said so far about quantitation of dosage-response relationships was concerned with all-or-none effects or effects that can be treated in this way. However, many responses of organisms are graded in character and need to be so reported. Such reports may take many forms, including tables and line and bar graphs, and represent the whole gamut of pharmacological effects. In some instances, the data may be treated mathematically. Some examples are given in the section on storage. However, graded responses do not lend themselves to neat quantitative tabulation such as may be applied, for example, to the LD 50 values of a series of compounds.

1.3.6 Dosage at the Tissue Level

A simple but profound change in pharmacology began in the 1940s when increased emphasis was placed on the importance of tissue levels of drugs. It had long been known that chemicals act at the cellular level, and the change in emphasis was conditioned largely by the rapidly increasing ability to carry out the necessary measurements which made it possible to relate the effectiveness of many drugs to their minimal plasma levels or, to be more exact, to minimal plasma levels of free compound. This critical index of the concentration available to cells made it possible to devise rational, nontoxic uses of several compounds that previously had been too slowly effective or too toxic to be practical.

The factors that can be involved in determining the plasma levels of free compounds have been reviewed *by* Brodie (1967) and by the National Academy of Sciences (1969).

Some factors other than dosage that control the concentration of foreign compounds available at the tissue level are discussed later.

Ultimately, the concentration of a drug or another (bio)chemical moiety at the site of action determines its toxicity, even for hit-and-run-type poisons whose continuous presence is not required. Therefore, the perfect EC 50 or LC 50 value would be to determine such a number directly in the target organ or tissue. However, that is often very difficult or impossible, because it requires either tissue biopsies or killing of the animals. Physiologically-based pharmacokinetic modeling eventually could become a very useful tool because it can predict tissue concentrations, but its accuracy is as yet unsatisfactory. In practice, toxicologists use surrogate measures for the concentration at the site of action. The most widely used, but not the best surrogate is the dose. The best surrogate is the plasma concentration of a chemical because the free fraction of the agent in plasma is in equilibrium with the free fraction in the target organ or tissue. It is often very difficult or even incompatible with the experimental design to obtain blood repeatedly from experimental animals. Therefore, the dose will remain a useful surrogate if properly qualified. The dose will be a poor surrogate for the tissue concentration of cadmium in testes after oral administration, because of very limited absorption from the gastrointestinal-tract, but it still will be proportional to it. Dermal absorption of some

chemicals is almost nil or very limited when high doses are applied to the skin. In such instances the dose will be a poor surrogate if at all for tissue concentrations. In inhalation studies the dose is again a good surrogate for the concentration at the site of action without regard for whether the lungs themselves or distant organs or tissues are the target(s) of toxicity for both types of compounds whose absorption is ventilation- or blood-flow-limited. Often the concentration of a volatile compound in the inhaled air is used as a surrogate. It is easy to convert an inhalation concentration to a dose if the body weight is known because the physiological parameters of respiration are well established in humans as well as in laboratory animals. There are some nearly perfect surrogates for target tissue levels such as the concentration of a water-soluble chemical in an aquarium or in an *in vitro* experiment.

1.3.7 Statistical Considerations

There are several good reference books on statistics applicable to problems in toxicology. These include volumes by Pearson and Hartley (1976), Mainland (1963), Steel and Torrie (1980), and Snedecor (1967). Useful statistical tables may be found in books by Beyer (1968) and Fisher and Yates (1963). A book designed specifically for toxicologists is that of Gad and Weil (1986) and a more recent one by Gad (2006).

This section is not intended as a substitute for the references just cited, and such books must be consulted by anyone interested in the mathematical details. This section does discuss some broad guidelines regarding (a) how many subjects are required for ordinary tests, (b) randomization of subjects, (c) selection of dosage levels, and (d) species considerations associated with the effects of small dosages.

1.3.7.1 Number of Subjects

As discussed in Section 1.3.1.1 and illustrated graphically in Fig. 1.4, the accuracy of statistical mea surement can be increased by running more tests under the same conditions.

Number of Independent Units Table 1.4 shows the difference that must appear between two equal groups to be significant at a level of $P = 0.05$, using groups of 50, 40, 30, 20, and 10 subjects, respectively. The simplest solution is that in which the effect under study does not occur in the controls. Inspection of the table shows that, when no controls are affected, there still must be 5 reactors (50%) among a group of 10 experimental subjects to achieve assurance that the difference between the two groups has not occurred by chance. If the groups are larger a smaller proportion of reactors is required. Thus, with groups of 50 subjects each, only 6 reactors (12%) are needed in the experimental group to indicate a statistical significance of difference when no controls react. Put another way, groups of 50 subjects each will be required to demonstrate dependably an effect that occurs in 12% of a very large population even when there are no reactors among the controls. Larger groups are required if events that occur at a lower frequency are to be demonstrated.

The second and succeeding lines of Table 1.4 are concerned with the situation in which there are reactors among the control group. If one group has a certain percentage of reactors, the other group must have a specified larger percentage for the difference to be significant. Thus, at least 50 animals per group would be necessary in order to give reasonable assurance that 26% incidence in one group and 10% incidence in the other group are in reality different.

Table 1.4 is intended to illustrate the principles just outlined. A more complete table suitable for guiding experimental work is provided in Mainland (1963).

Needless to say, even a mild clinical effect of a compound would be intolerable if it occurred in 1% of the general population who encountered traces of the material. If the effects were at all serious, an incidence of 1% among workers would be intolerable also. The solution of the problem from the standpoint of ordinary testing is to keep the limitations of precision in mind and to design experiments and select dosages in such a way that there will be one or more groups in which the parameter of interest approaches an incidence of 100%, while the incidence in the control is held very low. Interpretation of the results must be based not only on statistical consideration, but also on judgment regarding severity and reversibility of the effect under discussion, and the relevance of the test as a whole to the human situation.

Identity of Sampling Unit As critically reviewed by Weil (1970), it is an error to count individual subjects as statistical experimental units when these subjects are not randomly selected. For example, in studies of reproduction or teratogenesis, mothers (or litters) and not the number of offspring are the proper basis for statistical analysis. It is misleading to report the number of litters showing any malformation or, more precisely, the proportion of malformed young per litter. The reason, of course, is that the fate of any particular offspring is conditioned by the physiology of its mother and by the dosage she received.

Counting young rather than litters counts the same thing over and over. This tends to exaggerate the statistical significance of the results and may lead to the conclusion that observed differences are significant when they easily might occur by chance.

The same precaution must be observed in connection with studies of carcinogenicity started with newborn or infant animals.

Other types of unjustified grouping for statistical analysis are the combination of dosage groups, sexes, or strains

TABLE 1.4 Difference between Two Groups Necessary for Significance[a]

50 animals per group		40 animals per group		30 animals per group		20 animals per group		10 animals per group	
Least affected	Most affected	Least affected	Most affected	Least affected	Most affected	Least affected	Most affected	Least affected	Most affected
0	12	0	15	0	20	0	30	0	50
4	16	5	20	3 1/3	26 2/3	5	35	10	70
10	26	10	27 ½	10	36 2/3	10	45	20	80
20	38	20	42 ½	20	46 2/3	20	55	30	90
30	50	30	52 ½	30	56 2/3	30	65	40	100
40	60	40	62 ½	40	66 2/3	40	75	50	100
50	70	50	72 ½	50	76 2/3	50	85	60	—
60	80	60	82 ½	60	86 2/3	60	90	70	—
70	88	70	90	70	96 1/3	70	100	80	—
80	94	80	95	80	100	80	—	90	—
90	—	90	—	90	—	90	—		

[a]*From National Academy of Sciences (1960), by permission of the Academy. Differences are given as percentage incidence. $P = 0.05$.*

without testing the data statistically and finding that a particular combination is justified. A number of pitfalls in the applications of statistics have been discussed by a Task Force of Past Presidents (1982).

1.3.7.2 Randomization of Subjects

There is a possibility that error will be introduced into experiments through nonrandom selection of subjects, whether animals or people. For example, in selecting animals from a holding cage, there is a chance that the quieter ones will be taken first and the livelier ones caught later. If they are placed in groups as they are caught, there will be less tendency for successive groups to be more active, and the degree of activity may represent a basic physiological difference. The remedy is to give the animals temporary numbers as they are caught and then assign them to groups according to a table of random numbers. Such tables frequently are included in books of statistical tables. After the animals have been divided into groups, each one may be given a permanent identification number in serial order.

What has just been said about randomization concerns populations that either are considered homogeneous or exhibit variation impractical to control. Of course recognized variation may be made the object of controlled experimentation. For example, if differences involving sex are to be studied, the males and females must be segregated, after which subjects of each sex may be assigned randomly to appropriate groups. Because populations may lack homogeneity in many obvious ways, it is often desirable to limit a series of tests to animals of a preselected age or weight.

In working with a limited supply of subjects, it maybe better to ignore strict randomness in order to distribute some obvious variables among the different experimental groups. For example, if 30 men of widely different ages are to be placed in three dosage groups, it may be desirable to place their names on cards, arrange the cards in order by year and date of birth, and then deal the cards into three groups in the order 1, 2, 3, 3, 2, 1, 1, 3, 2, and so on. Although such a distribution is not random, it will eliminate bias. After a test has been run in this way, the data may be examined to see whether recognized variables affected the result. The results for each group may be plotted by age, or a coefficient of correlation may be calculated to see whether the outcome was significantly influenced by this variable. The results for one race may be compared to those for another, and other variables may be considered in turn.

1.3.7.3 Selection of Dosage Levels

Because the effects of chemicals expressed as probits usually form a straight line when plotted against the logarithm of dosage, it is best to choose a series of dosage levels that form a geometric progression. A factor of 2.0 (log interval of 0.3) is often used. More detailed information will be obtained if a smaller factor such as 1.26 (log interval of 0.1)

is used, especially in the region of the ED 50. Conversely, less (but sometimes sufficient) information can be obtained by testing dosage levels separated by a factor of 4 or more.

Selection of the general range of dosages to be studied is simply a matter of judgment supplemented by cautious trial. A number of methods for efficient use of small numbers of animals for determining ED 50 and LD 50 values are referenced in Section 1.3.1.1. Use of the logtime-logdosage curve for predicting the proper dosage range for tests involving repeated doses is discussed in Section 1.3.3.2.

These guidelines refer to quantitative studies of chemical effects that are agreed to exist, such as the lethality of any compound if given to any species at a sufficiently high dosage. However, for scientists who do not fully accept the dosage-response principle, a special problem in the choice of experimental dosage levels is presented by any study of a property that is thought to be possessed by some compounds but totally lacking in others. For example, use of the highest tolerated dosage is common in testing for carcinogenicity (Section 1.4.4). This is justified by those who practice it as providing the greatest statistical possibility of revealing a positive result. It is criticized by some others as providing an unrealistic result because it is based on dosage levels that people are unlikely to encounter and even experimental animals are unlikely to be able to detoxify and eliminate as effectively as they would the levels that occur in the environment. It seems likely that no solution to this dilemma will be reached except by relating the results of animal studies to epidemiological investigations.

The point is that no comparable problem in the choice of experimental dosage levels exists in connection with a study of a property such as lethality that is common to all compounds if administered at sufficient dosage. Thus a demonstration, whether in animals or humans, that table salt can he fatal does not pose any difficulty in evaluating its proper use. On the contrary, a demonstration that even a high dosage of a compound is carcinogenic in any species compromises the evaluation of it by those who do not fully accept the dosage-response principle. Although it seems likely that there are compounds that have and others that lack specific properties and although these differences may involve basic toxicological differences, such as the apparent lack of a third segment of the logtime-logdosage curve by carcinogens (Section 1.3.3.2), the main problem in evaluating compounds that do not have specific objectionable properties is one of established patterns of human thought, not of science. If this were not true, every potentially lethal compound would have to be banned, which means every and all drugs and other chemicals.

Statistical considerations did and still do play a predominant role in designing toxicological experiments. Indeed in the absence of a theory, which Hayes (1991) lamented in the discussion of the next section, proper statistical analysis is the only way to distinguish between chance occurrence and a cause-effect relationship usually characterized as 95% or higher confidence that two or more frequency distributions are truly different. Often, large biological variability is being made responsible for the lack of finding significant effects at some low dose in a defined population. The possibility that the standard toxicological protocols might be the primary reason for large variability for intra- and inter-laboratory results was not entertained, at least not from a theoretical point of view. Most ED or LD studies conducted in the past supposedly measured toxicity, when in fact they measured different combinations of intoxication and recovery. Because two or more variables were changing at the same time, the biological variability was greatly magnified. A third traditionally uncontrolled variable was due to the lumping of dead animals that died of different causes (mechanisms). Vehicle, formulation, volume, etc. are additional variables, which for the most part were much better controlled than these two much more important factors identified previously. The most reproducible studies were the ones conducted at toxicokinetic and/or toxicodynamic steady state, which "nature" sometimes happened to provide in the form of long kinetic and/or dynamic half-lives. For example, a compound with a kinetic or dynamic half-life of 1 h and a time to effect of 10 min will provide nearly pure toxicity data as does a chemical with a kinetic or dynamic half-life of 1 year and a time to effect of 60 days, because in both instances recovery will be relatively insignificant during the period of observation. However, as the ratio of kinetic or dynamic half-life to observation period becomes less and less favorable the contribution of recovery becomes greater and greater. It must be understood that just because an animal happens to die does not mean that the organism did not try to recover from the toxic insult. All definitive toxicological experiments have to be conducted under kinetic or dynamic steady state (ideal conditions) to determine the respective toxicological constants. Having done that makes the dose selection highly accurate according to $c \times t = k \times W$. Plotting $c \times t$ vs. W yields a straight line with the slope 1/k (Rozman, 2000a). Doing toxicological experiments "right" would have several advantages: They would require fewer animals, because of reduced variability. The experiments would become more reproducible; in fact, if conditions were kept ideal they would become entirely reproducible. The erroneous conclusion that the relative potency (structure activity by the same mechanism) of a chemical is dose-dependent (Fig. 1.5) would also be eliminated.

1.3.7.4 Effects of Small Dosages

Safety evaluation is much concerned with the effects of dosages just below and just above the threshold of observable effect, that is, with the *no-significant-effect level* and *lowest effect level* in practical experiments. After a consideration of these practical matters, the following paragraphs will present a discussion of what is known about the existence of thresholds and the beneficial effects of small dosages. These theoretical matters have clear implications for the probable

outcome of the quantitative study of the effects of dosages so small that extremely large groups of subjects are required for their meaningful investigation. Because of its difficulty and expense, this kind of quantitative study has rarely been carried out (Section 1.3.1.3), but further study would have tremendous theoretical and practical importance. The strictly toxicological study ought to be coordinated with biochemical investigations that could offer a reason for the statistical findings, no matter what they may be.

The No-Significant-Effect Level The concept of "no effect" is one of the most commonly employed in safety evaluation. The *no-observed-effect level* is the maximal or near maximal dosage level at which no difference from untreated or vehicle-treated controls is detected. It is not a level so far below any effective one that it is insignificant. Although the term "no effect" often is applied in connection with a dietary concentration, it is always in the context of the dosage administered. Although the term could be employed in connection with one or a few doses, it is employed mainly in connection with long-term tests. Use of the expression implies an organized study and careful observation, not the casual result of a test with some other purpose. Any study designed to reveal a dosage producing no effect should contain, in addition to a control group receiving no toxicant, one or more groups of subjects given larger dosages fully expected to produce measurable effects of the compound. Because such tests are almost always prolonged, particular attention must be given to the number of subjects assigned to each group so that the number available at the end of the study will be adequate to reach a statistically significant result (Section 1.3.7.1).

There are several reasons for placing the expression "no effect" in quotation marks. First, many studies reveal effects that are obvious, reproducible, and highly significant from a statistical standpoint, but of questionable biological significance. Depending on circumstances, an example might be partial inhibition of an easily regenerated enzyme. There is no substitute for judgment in toxicology. Second, as discussed in one of the following sections, the effects of small dosages of toxic compounds may be beneficial and thus qualitatively different from the effects of larger dosages. Third, failure to detect any effect by an elaborate scheme of testing does not exclude the possibility that an effect would have been detected if some other scheme had been used. Rapid progress has been made in chemical detection of toxicants or their metabolites. Analytical chemists already have achieved such skill that they easily measure biologically unimportant traces of several pesticides. Biochemists and physiologists are not far behind.

For these reasons, it is generally recognized that what is needed is a *no-significant-effect* or *no-observed-adverse-effect level* (NOAEL), that is, a level that causes no detectable injurious effect. There is no complete substitute for long-term tests, but increasing attention must be given to evaluating the biological significance of observed effects that involve no demonstrable injury to health. A toxicologist may do as much harm by unnecessarily condemning a compound as by failing to detect and prevent a real toxic hazard. Prophetic words indeed, albeit no attention was paid to them. In the contrary, Dr. Hayes' concern and fear has become today common practice among many toxicologists.

The Lowest Effect Level as a Practical Toxicological Measurement As already mentioned, it usually is impractical to use such a large number of subjects per group that the possibility of a rare ($<1\%$) but highly undesirable effect, such as neurotoxicity or carcinogenesis, can be excluded in the lower dosage range. This problem is not as important as it may appear at first, because the frequency of an effect can be increased by increasing the dosage (just one reason for using injurious dosages in long-term studies). Thus, the existence of a highly undesirable effect of a particular compound can be taken into account in the selection of a safety factor. A more serious objection from a purely scientific standpoint is the imprecision of finding the highest no-significant-effect level. From a statistical point of view, it would be preferable to employ for safety evaluation the lowest effect level, that is, the smallest one at which a meaningful, injurious effect or even a relevant, harmless effect is detected. This implies that (a) the no-significant-effect level is determined in order to put a limit on what is meant by the smallest effect level, but (b) the more objective, positive finding is used for safety evaluation, and (c) the nature of any injurious effect observed will be taken into account in choosing a safety factor.

Threshold Levels as Biological Facts The practical difficulty in establishing a "no-effect" level for a particular compound using a manageable number of experimental animals and the more complex problem of extrapolating a safe level for humans must not be permitted to obscure the fact that thresholds do exist. The toxicologist faced with a single limited experiment would be wise to recall the impossibility of proving a negative. The logician faced with a complete lack of supporting evidence would be wise not to press pure logic too far and conclude that no threshold exists so that even a single molecule may represent a hazard. As pointed out by Friedman (1973), the question of the existence of a threshold is a problem of biology, not of mathematics or of probability. In not a single instance has the absence of a threshold been demonstrated. On the contrary, concentrations are known at which compounds with the highest biological activity are inactive.

For example, as described by Friedman (1973), limitation of vitamin A intake to 10% of the minimal required dosage leads to severe deficiency disease, yet this constitutes a dosage of 3.6×10^{15} molecules per kilogram of body weight or a concentration of 6×10^{-9} M in the body. In a similar way, ineffective levels of vitamin D and vitamin B_{12} (for which the daily requirements are only $10\,\mu$g/day and

1 μg/day, respectively) are 4×10^{-11} M and 1×10^{-12} M. By conservative estimates, post-menopausal women and adult men have 1.5×10^{12} molecules of estradiol per kilogram of body weight or the equivalent of 2.6×10^{-12} M.

For 2,3,7,8-tetrachlorodibenzodioxin (TCDD), reported to have an LD 50 value of 0.0006 mg/kg in guinea pigs, a harmless dose would still produce a concentration of 1.9×10^{-10}M. Botulinum toxin, for which activity described in terms of molecules has long been common, would produce absolutely no effect in mice at a dosage of 4.2×10^{7} molecules per kilogram or a concentration in the mouse of 7×10^{-17}M, assuming a molecular weight of 900,000.

Values of the same orders of magnitude apply to some carcinogens. An ineffective amount of aflatoxin in the rat consists of a dosage of 9.6×10^{-12} molecules per kilogram and a concentration of 1.6×10^{-11} M. However, many strong carcinogens are less potent. For 1, 2, 5, 6-dibenzanthracene, methylcholanthrene, and 3,4-benzpyrene administered by different routes, the ineffective concentration in the body ranges from 1×10^{-8} to 1×10^{-1} M.

The limiting level is even higher for compounds that are not inherently very active. For example, an ineffective amount of Aramite as a carcinogen is a concentration of 3×10^{-1} M.

Hutchinson (1964) and later Dinman (1972) suggested that 10^4 molecules per cell is the limiting concentration for biological activity, whether pharmacological or injurious. As pointed out by Friedman (1973), there are about 6×10^{13} cells in a 70-kg human body, from which it follows that the suggested limiting level for activity is 8.6×10^{15} molecules per kilogram or about 1×10^{-8} M. The demonstrated no effect levels for vitamin D, vitamin B 12 and estradiol (10^{-11}–10^{-12} M) are so low that the corresponding thresholds may be somewhat lower than the limiting level of 1×10^{-8} just discussed. The same reasoning applies to the thresholds of toxic action of TCDD and botulinum toxin and the threshold of carcinogenic action of aflatoxin. Further evidence that the limiting concentration for the inactivity of a few highly active compounds is less than 10^{-8} M is the report that certain pheromones have thresholds on the order of 1×10^{-12} M. These values do not prove that the limiting concentration is even less than 10^{-8} M in susceptible cells, because some compounds, such as botulinum toxin, have extremely high affinity for the cells on which they act, and their distribution in the body at critical dosage may be very uneven.

However, exactly what concentration is limiting is far less important than the fact that thresholds do exist even for the most active compounds. No one doubts that the existence of deficiency conditions proves that minimal or threshold concentrations of vitamins, hormones, and other beneficial compounds are required for proper action. There is no chemical or other scientific reason to suppose that there is an inherent, fundamental difference in the dosage-response relationship of injurious compounds.

Predicting the effects of small doses has been one of the core problems of toxicology (Rozman and Doull, 1998). Knowledgeable toxicologists were always aware of the existence of biological threshold doses, which would not cause any response in a given biological system. However, because of a statistical rather than a theoretical or biological view of the dose-response and because of a lack of considerations of time as a quantifiable variable of toxicity, a definition of a threshold in toxicology remained elusive. As long as one looks at toxicity only in terms of dose-responses it is logical to arrive at NO(A)ELs and LO(A)ELs as starting points for safety and risk assessments. The problem is that a NOEL is fuzzy if only a few doses have been used, often one or more orders of magnitude apart. The LOEL is less fuzzy because the magnitude of the effect provides an estimate of how far away a NOEL might be for a given population size. Here toxicology has encountered a thus far insurmountable difference of opinion among its practitioners with no resolution in sight. There are those who believe that some dose-responses are so shallow at the lower end that the terminal slope is essentially linear, which corresponds to a very large standard deviation in terms of frequency distribution of a normally distributed effect. Others point out that the concept of the maximum tolerated dose (MTD) prevents the experimental exploration of a full dose-response and that most of the currently available long-term studies represent truncated dose responses limited to the low dose end of the dose-response. A lack of theoretical considerations is at the heart of this difference in opinion. Most chronic studies (acute experiments were argued in Section 1.2.2.1) entail intermittent administration of chemicals with different periods of recovery between exposure episodes. If the kinetic-dynamic half-life (as is the case for most chemicals) of a compound is very short then there will be slow if any accumulation of injury because of significant or complete intermittent recovery. Thus, most of the chronic toxicological experiments of the past measured various combinations of intoxication and recovery. Dependent on the ratio of the two (and on additional "hidden" variables) the frequency distribution and hence the dose response becomes flatter and flatter because individuals almost never belong to the same normal distribution in terms of both injury and recovery. For example, the gene responsible for producing acetylcholinesterase (AChE) in the respiratory center is different from the genes producing carboxyesterases released into blood. Individuals could have a high synthesis rate of AChE in the pons and medulla but low production rate of carboxyesterases or vice versa in any quantitative ratio. Susceptibility of an organism to organophosphate toxicity depends on the carboxyesterase pool (detoxification) but recovery from the intoxication is determined by the rate of AChE production in the critical brain region. The conclusion from these considerations is that to measure true (pure) toxicity one must conduct an experiment under kinetic-dynamic steady state, which often

is possible only by continuous exposure until the occurrence of a well-defined effect. Although it might be often impractical to live up to this ideal condition, experimentalists must be aware of it to avoid claiming exceptions from the theory of toxicology when, in fact, they are not controlling some variables. Experiments conducted under ideal conditions controlling all variables but the dose and one timescale (that of intoxication) will always yield $c \times t = k$ under isoeffective conditions or $c \times t = k \times W$ under isodosic or isotemporal conditions. It is from this relationship that a true LO(A)EL can be determined by substituting for time (*t)* the maximum life span of a given species. In its immediate vicinity is the NO(A)EL in terms of threshold dose which will cause no effect whatsoever in a lifetime, for a given population,

$$c_{threshold} = \frac{k \times W}{t_{lifespan}} \quad \text{(isodosic and isotemporal)}$$

$$c_{threshold} = \frac{k}{t_{lifespan}} \quad \text{(isoeffective)}$$

Beneficial Effects of Small Dosages Schulz (1888) may have been the first to observe stimulation from very low concentrations of poisons. He investigated the effect of mercuric chloride,iodine, bromine, arsenous acid, chromic acid, formic acid, and salicylic acid on yeast and concluded that, when sufficiently diluted, all of them can increase the vitality of yeast over a longer or shorter period of time. Only a few years later the bacteriologist Hueppe (1896) stated the rule that has come to bear his name. "Every substance that kills and destroys protoplasm in certain concentrations inhibits development in lower concentrations, but acts as a stimulus and increases the potential of life at even lower concentrations beyond a point of neutrality." In stating this principle, Hueppe mentioned certain apparent exceptions. He also acknowledged the independent discovery of the rule by Arndt and Schulz.

A special universally accepted instance of the beneficial effects of small dosages involves the reactions of plants to what are now called essential trace elements. A plant cannot live if even a single one of these metals or metalloids is absent from the medium in which the plant is grown, but an excess of any one of them is injurious. This is the law of optimal nutritive concentration. Recognition of it must be credited to Gabriel Bertrand even though he may never have stated it concisely in a publication. The relationship of beneficial and harmful effects of trace elements was implied in the discussion that followed his presentation on "complementary nutrients" at the Fifth International Congress of Applied Chemistry held in 1903 in Berlin (Bertrand, 1903). Personal communications from his son, Dr. Didier Bertrand, and one of his students, Dr. Rene Truhaut, established that Gabriel Bertrand presented the law of optimal nutritive concentration in his course at the Sorbonne from 1908 through 1930. Later, M. D. Bertrand (1962a, 1962b, 1969) generalized the law and expressed it in a mathematical form.

As reviewed by Townsend and Luckey (1960) and in a very different way by Smyth (1967), evidence has continued to accumulate that small dosages of many compounds are not injurious, but beneficial. Townsend and Luckey tabulated many examples from the pharmacological literature. Smyth offered several original examples of benefits from small doses of toxicants. The benefit may be substantial and include increased rate of growth, greater fertility, and prolonged life span. The phenomenon, or variants of it, has received different names. Thus the noun "hormesis" and the adjective "hormetic" were proposed for the stimulatory action of a subinhibitory amount of a toxicant (Southam and Ehrlich, 1943). The term "hormoligosis" (from the Greek *hormao,* rouse or set in motion, and *oligos,* small) was proposed (Luckey, 1956) to indicate the more general process by which a small amount of anything, regardless of its toxicity, produces stimulation. The same author used the term "hormoligant" to indicate something that stimulates when given in a small amount. The term "sufficient challenge" introduced by Smyth (1967) refers to the entire range of phenomena and emphasizes the need of the organism for some measure of stress, whether it be a small amount of poison, a small amount of radiation, or early, immunizing infection. In fact, he points out that he took the term from Toynbee's concept of "sufficient but not overpowering challenge" in connection with human history.

There is a tendency to take for granted the beneficial effects of small amounts of certain classes of compounds, which we call drugs, nutrients, or growth promoters, and to ignore completely the beneficial effects of small amounts of other materials, some of which we call poisons. The difference depends largely on our supposed understanding of their actions.

Since antiquity, the use of therapeutic drugs has seemed reasonable to people. Thus we "understand" the benefits from this one class of materials that are clearly toxic at higher doses. A nutritional mechanism for the stimulation produced by low concentrations of certain toxic substances offers another basis for understanding. A number of minerals, vitamins, amino acids, and fatty acids are known to be essential to animals. The fact that excessive intake of some of them, notably several of the metals and vitamins A and D, has led to cases of human poisoning, has not detracted from acceptance of their benefit. The discovery of the induction of processing enzymes, especially the mixed function oxidases of the liver, has added a third means of understanding the benefit of small amounts of some drugs, pesticides, and other chemicals. Finally, the effectiveness of growth-promoting feed additives is understood in a somewhat different sense. There is little or no reason to think that the effectiveness of arsenilic acid and various antibiotic feed additives depends on any nutritive

value. Their mode of action when given in the usual homeopathic dosages is suspected only to be related to action on the microflora, but their ability to make chickens, pigs, and calves grow faster is inescapable. In this case understanding is mainly in terms of commercial success.

There is a tendency to ignore the beneficial effects of small doses of toxic compounds unless they are understood in terms of therapeutic action, nutritional requirement, growth promotion, or perhaps enzyme induction. In the latter case, there is some ambivalence and tendency to view adaptive change as evidence of injury. A few scientists have the courage to see in the induction of enzymes an evidence of adaptation at the molecular level. Toxicologists should combat all bias. The existence of a phenomenon does not depend on our understanding of it. Statistically established evidence of benefit from small dosages ought to be viewed just as objectively as statistically established evidence of injury from larger dosages. This statement is not meant to underestimate the importance of increasing our basic understanding: it is a plea to explore widely and to accept facts even when they appear contradictory.

Most compounds have two or more modes of action which reach expression at different, though perhaps overlapping dosage levels. Possession of more than one mode of action certainly opens the possibility that low dosages of a compound will be beneficial rather than merely harmless. However, both benefit and harm may be associated but are not necessarily associated with a single mode of action. Most, but not all, of the side effects of drugs are excessive expressions of their therapeutic actions and the result of overdosage.

It is a general principle that excessive dosages of beneficial compounds are always toxic. It may be that the converse is also true, for the possibility cannot be excluded that sufficiently small dosages of toxic compounds are always beneficial in some living system: each apparent exception may be merely the result of failure to test a particular material under appropriate conditions.

Hayes (1991) was fully aware of the widespread presence of beneficial effects of small doses of chemicals such as drugs, essential nutrients, and other hormetic agents. Calabrese and Baldwin (2001a) evaluated the literature and found hundreds of cases of clear (dose-dependent) hormetic effects of chemicals that otherwise were only looked upon as toxicants. In contrast to Hayes (1991), who took an integrated view of the beneficial effects of chemicals (with which we agree), Calabrese and Baldwin (2001a) assumed a viewpoint of conceptual fragmentation by excluding drugs and nutrients from the group of chemicals having hormetic effects. The β curve suggested by Calabrese and Baldwin (2001b) to conceptualize hormesis is also problematic. It is implausible from the biological point of view that a chemical can cause both an increase and a decrease in an effect by the same mechanism (Rozman and Doull, 1999). It is more reasonable to extend Hayes' view to all chemicals having the potential of exerting beneficial (hormetic) effects in small doses, although often the benefit may be immeasurably small. Increasing doses will neutralize the benefit by a different mechanism and eventually lead to toxicity by the same or a still different mode of action. Thus the β curve is an attempt to combine two or three parallel or nonparallel dose responses into a single curve. It has no foundation in the principles of toxicology. Homeopathic claims of small doses of naturally occurring or man-made chemicals are equally incompatible with the principles of toxicology unless supported by clear dose- and time-response relationships.

In fact, the principles of toxicology (large doses) and pharmacology (small doses) are highly compatible with Toynbee's view (see Smyth, 1967, and Hayes above) of successful and unsuccessful societies in historical context, which means in the dimension of time. A "sufficient, but not overpowering challenge" which increases the fitness of a society to respond to larger challenges is similar to small doses, which enhance the fitness (adaptation) of an individual to respond to subsequent higher (toxic) doses. However, an overpowering challenge very much like a very large dose results in the demise of both a society and an individual.

Logprobit Model and Quantitative Study of the Effects of Small Dosages It is implied by the logprobit curve for dosage-response that an effect occurring in a low proportion of a population, for example, an average of 1 in 10,000, is merely the result of a smaller dosage than what would produce the same effect in a high proportion of the same population. The number of subjects necessary to measure the effect of a truly small dosage is very great unless this effect is qualitatively different from that produced by a large dosage.

If a small dosage produces a beneficial effect compared with the control, it may be possible to establish this fact with no more experimental subjects than are required to show that a larger dosage is harmful, and the presence of a positive benefit may exclude the possibility of a hypothetical injury from the same dosage. This principle is illustrated by facts regarding selenium, a trace element essential to life. Using groups of only eight animals each, G. Siami (personal communication, Siami, 1971) showed clearly that rats fed a dietary supplement of 1 ppm selenium grew better than rats fed the same regular commercial rat feed which contained only 0.15 ppm of the element. Rats receiving a supplement of 2 ppm also grew better than the controls but not as well as those receiving the 1-ppm supplement. Rats receiving 3-ppm supplement showed definite toxicity, including liver cirrhosis, and those receiving 5 ppm of selenium died in only 5 weeks. Thus, the presence of a positive benefit at 2 ppm excludes, within the limits of the experiment, the possibility of injury from this exposure even though 3 ppm was distinctly injurious.

If the effects of different dosages are qualitatively identical and if it requires groups of 10 subjects to identify

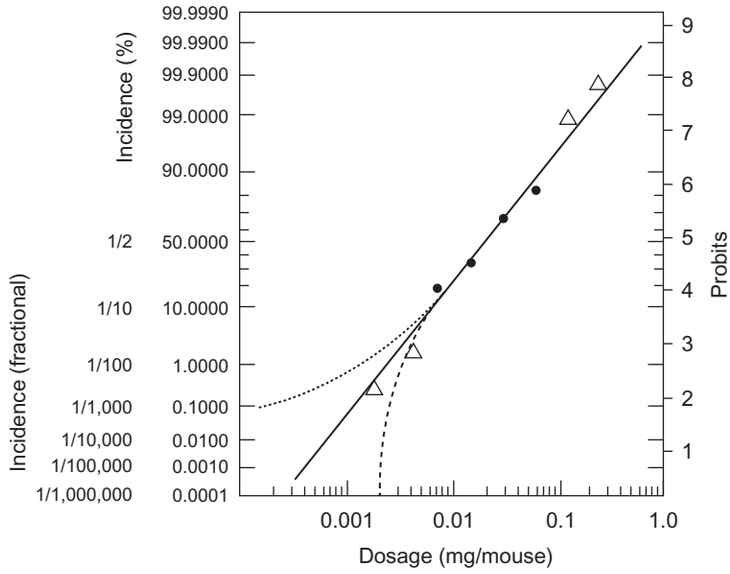

FIGURE 1.13 Incidence of tumors in mice following a single subcutaneous dose of 20-methylcholanthrene: (●) observed values; (△) values adjusted from 0 or 100% by the method of Litchfield and Wilcoxon (1949). Hypothetical deviations from the straight line logprobit relationship are ahown by a dotted and a dashed line, as discussed in the text. Data from Bryan and Shimkin (1943).

with acceptable precision the dosage that, on the average, causes an event once in every two chances (ED 50), then it would require groups of 500 subjects to find with the same precision the dosage that, on the average, causes an event once in 100 chances (ED 1). For an event affecting 1 in 10,000 (ED 0.01), groups of 50,000 subjects each would be required. Such large groups of subjects are required to measure the effects of small dosages directly that this measurement is entirely impractical in connection with routine toxicological testing. In fact, such studies have been done only rarely.

Reasons for not doing statistical studies of small dosages include (a) their great expense, (b) the technical difficulty of preventing or even recognizing effects caused by uncontrolled variables, and (c) the fact that tests employing a reasonable number of animals per group give results of practical value entirely suitable for determining safe levels of human exposure. The safety factor [whether in the form of a fraction of the no-significant-effect level or the lowest effect level or in the form of standard deviations] removes the acceptable dosage one or two orders of magnitude from the lowest dosage tested experimentally and, therefore, far from the area of danger.

The main reason for doing thorough, direct, statistical studies of the effects of small dosages is that we lack that knowledge. For practical purposes, we can compensate for our ignorance by using safety factors that exceed the degree of uncertainty involved. However, there might be practical as well as theoretical value in exploring with precision the effects of small dosages. Sound information would reveal the limits of variability and thus indicate more accurately what safety factors really are needed-a practical result, greatly to be desired.

The linear logprobit dosage-response curve was developed on the basis of observed facts but also involves the theoretical assumption that response to dosage follows a lognormal distribution. The logprobit model fits the observed facts and greatly facilitates their orderly study and presentation (Section 1.3.1). However, there is no way to be sure that the model fits the facts in an area where almost no measurements have been made.

Hypothetically, the logprobit curve could deviate from a straight line either upward or downward in the low dosage region. Any true deviation would indicate that the distribution for a complete range of dosage levels is not lognormal under the conditions tested. A deviation in the low dosage level would not indicate that the distribution for dosages near the ED 50 level is not essentially lognormal, for the conditions relative to this level and to low dosages may be qualitatively different.

The direction of deviation would depend on the nature of the physiological factor responsible for nonnormal distribution. If there is no threshold for the effect under study so that no dosage, no matter how small, is totally without the effect measured, then the line must deviate upward and approach a horizontal direction in such a way that it will pass through absolute zero located an infinite number of logarithmic cycles to the left, There is no example to illustrate this condition,

but it must be considered from the standpoint of logic. If a statistically valid example were found, it would indicate either an unsuspected variable in the experiment or the existence of a yet undiscovered principle of toxicological action.

If the threshold for the effect under study does not lie in the lognormal distribution but at a dosage higher than that predicted by this distribution, then the logprobit line must deviate downward and approach a vertical direction. There are literally thousands of possible examples of this situation. As often as not, the lower part of a logprobit curve is made up of points adjusted from zero values. The adjustment is made in the faith that a lognormal distribution is involved, even though some of the observed zero values are based on large enough groups of subjects to be statistically likely to give values higher than zero. Of course a number of examples will have to be tested at low dosage levels and with very large groups of subjects before a conclusion about the existence and eventually the frequency of nonlognormal distribution may be reached. If this kind of nonlognormal distribution were demonstrated, the statistical model would have to be adjusted, but no new principle of toxicological action would be indicated. The mechanism would have to be learned in each instance, but possibilities are known. For example, the downward flexure of the curve might correspond to the transition between low dosages metabolized easily by one pathway and higher dosages that overload the normal pathway and involve other pathways also.

It appears likely that, if the kind of deviation from lognormal distribution under discussion exists, it is related more often to the ability of an enzyme to cope successfully with low dosage levels than it is related to beneficial effects. Unlike the situation with selenium mentioned earlier, beneficial dosages of many essential elements are one or more orders of magnitude smaller than the smallest dosage observed to be injurious.

Figure 1.13 illustrates the matters that have just been discussed. The figure shows the proportion of different groups of mice that developed tumors at the site of injection of a single subcutaneous dose of 20-methylcholanthrene as reported by Bryan and Shimkin (1943). It may be seen that the observed values in the area of the ED 50 correspond well to the expected lognormal distribution. The adjusted values for low dosage levels also correspond well, for they have been made to do so. However, each of these adjusted values for low dosages is derived from an observed value of zero. In Fig. l.13, the straight line required by the observed values in the area of the ED 50 has been extended at each end as required by a lognormal distribution of all values. However, in the low dosage area, an upward flexure of the curve has been inserted to illustrate a no-threshold relationship, and a downward flexure has been inserted to illustrate the opposite deviation from a lognormal distribution.

Similar reasoning could be applied to the upper portion of the curve, but it would be of no real interest from the standpoint of safety evaluation.

Statistics is a legitimate and useful tool to describe incompletely understood phenomena as discussed by Hayes (1991). However, overuse of statistics as it occurred and still is occurring in toxicology hampers the development of a theory which represents the only true epistemological gain for a discipline. In the mean time, a large number of statistical methods other than the logprobit method have been proposed (Holland and Sielken, 1993). Yet safety and risk predictions have not become more accurate: they just keep appearing more and more sophisticated mathematically. Models are only as good as their underlying assumptions, which in the absence of a theory are just slightly or not at all better than superstition.

Let us now look at the Hayes (1991) example (methylcholanthrene and skin cancer) in light of the theory of toxicology. The kinetics of polycyclic aromatic hydrocarbons has not been studied much, which is surprising considering the enormous amount of work that went into studying their carcinogenicity. Limited data (ATSDR, 1995b) indicate that their biological half-life is on the order of hours to a day, depending on the route of administration. However, a single subcutaneous dose rate (dose) of 20-methylcholanthrene caused up to 80% incidence of cancer at the site of injection, which indicates that this compound's toxicity is dominated by dynamic rather than kinetic-dynamic processes. This is compatible with a long half-life of the postulated DNA-adduct in analogy to benzo[a]pyrene. The actual dose range for measurable incidence encompasses almost 2 orders of magnitude indicative of a shallow slope (Fig. 1.13) associated with large uncertainty as to the shape of the curve in the low dose region. A single dose rate (dose) study in combination with a very long observation period is far from ideal conditions because the organism is not at steady state with regard to the injury. Rather the animals are continuously repairing the damage while the cancer is developing. Those animals receiving lower doses have more time to repair the damage, which further increases variability, because some animals actually do reduce the DNA damage to an extent, which does not necessitate the development of cancer within their natural life span. The major conclusion to be drawn from these considerations is that increased variability in the low dose region is due to the concurrent measurement of toxicity (cancer) and recovery.

The experiment which does not allow for recovery to occur has also been conducted (Horton and Denman, 1955). Continuous exposure of mice to methylcholanthrene caused cancer in a highly predictable manner with a $c \times t = 126.7 \pm 11.1$ mg/kg/week, representing a mere 4.2% variability over a very steep dose (total dose) range of 4.0–15.1 mg/kg. For a compound with a short half-life like methylcholanthrene only repeated exposure will result in the animals attaining toxicodynamic steady state after about 4 recovery half-lives after which $c \times t = k$ occurs according to a rectangular geometry. Mistaking the (daily) dose rate for the dose, when, in fact, the dose is always the sum

of (daily) dose rates, has been one of the most damaging assumptions toxicologists ever made, because it makes the $c \times t = k$ concept disappear as was the case with methylcholanthrene. It must be recognized that assuming that a daily dose rate is a dose amounts to ignoring time, in that animals receiving lower doses live a lot longer than those given high doses and therefore the daily dose rate artificially and with no theoretical legitimacy flattens the slope of the dose response, leading to additional variability in the low dose region. The fact that no mouse lives beyond 1500 days allows one to conduct a highly accurate biological safety-risk assessment by substituting $c_{\text{threshold}} \times 1500 = 126.7 \pm 11.1$ yielding a dose for $c_{\text{threshold}} = 0.85$ mg/kg or a daily dose rate of 0.004 mg/kg/week. The uncertainty about this number arising as a result of the variability of k (4.2%) is very small (Section 1.3.7.4).

In other instances, the relationship between applied dose and the time to tumor was unsatisfactory even when plotting dose rather than dose rate (daily dose) vs time to effect as with benzo[a]pyrene (Poel, 1959). As discussed previously in Sections 1.1.3 and 1.1.4. there are two important variables that need to be controlled or otherwise the $c \times t = k$ relationship is lost. One is the exact definition of the effect such as time to onset of a response (first tumor) or time to 100% response (single tumor) because the definition of ideal conditions requires that experiments examining dose-time relationships must he conducted under isoeffective conditions. This was not the case with benzo[a]pyrene, but with methylcholanthrene it appears to have been the case. The other problematic variable in toxicity studies using dermal application is that the applied dose is often a poor surrogate of the dose (concentration at site of action), because after saturation of the flux through a defined skin surface, there is no more proportionality between applied dose and systemic dose. It is quite apparent that neither variable was well-controlled in the study with benzo[a]pyrene and, perhaps fortuitously, both were controlled in the experiment with methylcholanthrene.

This discussion illustrates that improvements in safety-risk assessment are not going to arise out of more statistical sophistication, but out of conducting toxicological experiments under ideal conditions (kinetic-dynamic steady state) and then understanding how departures from ideal conditions influence the slope of the dose-time- responses and their variability at low doses and short times.

Other Models of Dosage-Response Relationships Other ways of describing dosage-response relationships include the logprobit and one-hit models. They give results very similar to those for the logprobit method of analysis at the ED 50 level and even in some instances at the ED 16 level. However, the logprobit and one-hit models predict effects of low dosages quite different from those predicted by the logprobit model. The tails of the distributions differ widely even though the central portions are similar (Mantel, 1963). Thus, the degree of reduction of the dosage that produces tumors in 1% of subjects necessary to achieve virtual safety (defined as the production of only one tumor in 100 million subjects) differs widely for the three models, namely, 1/100 for the logprobit model, 1/100,000 for the logistic model, and 1/1,000,000, for the one-hit model (Mantel and Bryan, 1961). In other words, the curves for these other models deviate upward and to the left compared with the linear logprobit relationship.

The objective studies of the effects of small dosages discussed in the foregoing section would test all three models simultaneously. It is not necessary to test extremely low dosages but only to learn whether the observed points deviate from the logprobit relationship, and if so, in what direction. For example, in Fig. 1.13 for groups of 1000 the estimated number of mice developing tumors following a dosage of 0.0039 mg per mouse would be 50 for a no-threshold model, 35 for a lognormal distribution, and 18 if a threshold is involved. After a dosage of 0.00195 mg per mouse, the estimates would be 20, 5, and 0.001 (i.e., zero) mice, respectively. Certainly it ought to be possible to distinguish values of these magnitudes with only a relatively few repetitions of an experiment.

Chemical Basis of Thresholds in Dosage-Response Relationships A great deal is known about the biotransformation of foreign compounds and also about the effects of toxic substances on the otherwise normal metabolism of the body. This knowledge, explains many toxic actions and dosage-response relationships. However, the biochemical basis of the effects of small dosages is poorly explored, just as their dosage-response relationships are poorly studied.

It is clear in a general way that thresholds involve dosage but are not necessarily directly proportional to it, and that they are conditioned by the ability of the body to repair some injuries.

Sufficiently small doses are without detectable effect. The effects of somewhat larger doses may be harmless in themselves and completely repaired before the next dose is received. This relationship is well illustrated by the action of inhibitors on enzymes when administered at rates that do not cause illness.

What is not clear is the identity and relative importance of mechanisms that do not correspond directly with differences in dosage and, in this sense, may be regarded as qualitative differences. It is often speculated that small doses are biotransformed by normal pathways without taxing them, but that larger doses saturate these pathways, flood others, and thus interfere with endogenous metabolism. Unfortunately, details frequently are lacking, but there are notable exceptions, some of which are discussed in the following paragraphs.

Capacity for biotransformation may explain the presence of a threshold. Furthermore, biotransformation may be one mechanism of repair as is illustrated by the classical

example of cyanide poisoning. Prompt metabolism of the cyanide ion to the much less toxic thiocyanate ion serves to prevent a combination of cyanide with cytochrome oxidase (the biochemical lesion in this instance). However, if the lesion already has formed, metabolism of cyanide to thiocyanate helps to establish a gradient that favors release of cyanide from combination with cytochrome oxidase and in this way promotes repair of the biochemical lesion. The tremendous efficiency of the metabolism of small doses of cyanide to thiocyanate explains why we are able to withstand the small amounts of cyanide we receive daily from food and other sources. However, above a certain threshold, cyanide is dangerous. In this instance, the limiting factor is not the capacity of the enzyme thiosulfate sulfotransferase but the immediate availability of sulfur to form thiocyanate. Moderate doses of cyanide cannot be metabolized efficiently because the sulfur compounds ordinarily used for this purpose are limited in availability. That the limitation of sulfur is, in fact, the reason for the threshold above which cyanide becomes dangerous is demonstrated by the fact that the threshold is moved upward if a suitable source of sulfur is furnished. The difference can be measured best not in terms of the threshold itself, but in terms of the LD 50. It was shown by Way *et al*. (1966) that the LD 50 of potassium cyanide can be shifted from 9 to 33 mg/kg merely by supplying sodium thiosulfate (see Way *et al*., 1966).

Glyoxylate is significantly more toxic than ethylene glycol, of which it is a metabolite, and it probably is largely responsible for the toxicity of the parent compound. When the dosage of glyoxylate to monkeys was reduced from 500 to 60 mg/kg, the proportion excreted unchanged was reduced from a maximum of 59% to a maximum of 1.5% and the proportion metabolized to carbon dioxide increased. Thus the kidney, which is specifically susceptible to injury by ethylene glycol and glyoxylate, is protected to a disproportionate degree by the metabolism of low dosages as compared with the metabolism of high dosages (McChesney *et al*., 1972).

Liver glutathione (GSH) has a relation to the toxicity of bromobenzene somewhat analogous to that of available sulfur to the toxicity of cyanide. There is a close relationship between the covalent binding of halogenated benzenes and their ability to cause necrosis of the liver. However, covalent binding of bromobenzene metabolites to mouse liver protein remains low until a critical dosage of 1.20–2.15 mmol/kg is reached. At dosages of 2.15 and 4.06 mmol/kg (which produces minimal and extensive toxicity, respectively), the rate of covalent binding is not only high, but is over twice what would be predicted by extrapolation of the rates for lower, nontoxic dosages (Reid and Krishna, 1973). Bromobenzene, or especially its epoxide, depletes liver glutathione in the process of forming a mercapturic acid. Little covalent binding of bromobenzene metabolites occurs while the supply of GSH is adequate and mercapturic acid is being formed, but considerable covalent binding occurs when 90% of the liver GSH is lost and little mercapturic acid can be formed (Jollow *et al*., 1974). Anything that reduces liver GSH (even though harmless in itself) makes the liver more susceptible to injury by bromobenzene. Finally, as outlined in Section 1.1.4, bromobenzene is capable of metabolism by different pathways and the protection is at least partially independent of GSH availability. Thus the metabolism of bromobenzene is complex, but the available facts all help to explain its disproportionate increase in toxicity above a threshold.

As reviewed by Gillette (1973), the toxicity of acetaminophen shows a disproportionate increase above a threshold and this relationship depends at least in part on the availability of liver GSH.

Another example involves the onset of toxicity at dosage levels that exceed the metabolic capacity of the liver. Golberg *et al*. (1967) found that 2,4,6-tri-tertbutylphenol at a dosage of 0.5 mg/kg/day for 6 days induced hepatic processing enzymes, and the efficiency of the enzymes was such that the concentration of the compound in the liver was distinguishable after dosages of 10, 25, and 50 mg/kg/day. However, a dosage of 75 mg/kg/day produced an approximately eightfold increase in average liver storage, and it was only at this threshold dosage range of 50–75 mg/kg/day that toxicity as indicated by histopathological changes in the liver first appeared. In a study of a series of substituted phenols, it was found that liver processing enzymes invariably were induced by dosages lower than those required to alter the activity of liver microsomal phosphatases or to produce histopathological change (Golberg *et al*., 1967).

Stokinger (1953) reviewed evidence that the tissue distributions of beryllium, silver, iron, and iodine differ, sometimes greatly, according to dosage. His interest was focused on the serious errors that may be introduced by extrapolating the results of the storage of small tracer doses to the storage of a therapeutic or even toxic dose. Although his interest was in tissue distribution rather than toxicity *per se,* he noted that one might expect to find a different pattern of toxic manifestations solely because of the different amounts of the toxic agents in various organ sites. Stokinger suggested several mechanisms governing the distribution of the elements listed: (a) dosage-dependent formation of colloidal hydroxides such as those of beryllium, which are then phagocytized by cells of the reticuloendothelial system, (b) formation of complexes with serum proteins or other colloids (e.g., complexes of silver), which are then phagocytized, and (c) complex physiological regulation such as that of iodine or of iron.

As discussed earlier, nonlinear dosage-dependent differences in the toxicity of foreign organic compounds, unlike those of the elements, are likely to depend on other mechanisms, namely, (a) biotransformation and (b) biorepair. There may be other mechanisms also, for the subject has been studied inadequately.

It has been pointed out that the only beneficial effects of small dosages of toxic substances that now are accepted generally are those that are understood. This uncompromising demand for intellectual justification is admirable. It is clear that the concept of threshold and the observed beneficial effects of at least some small dosages will be more readily accepted if their biochemical basis is elucidated more completely. Studies of dosage-related biochemistry ought to go hand in hand with statistical studies of the clinical effects of small dosages.

Discussion In the exposition of statistical studies involving large groups of animals in Section 1.3.1.3, the ED 01 study of the carcinogen 2-AAF was considered. No biochemical study has explored whether there is any difference between the way the compound is metabolized at dietary levels below 60 ppm and the way it is metabolized at dietary levels of 60 ppm and above that would explain the form of the curves for bladder cancer that we have discussed, Similarly, there are no biochemical studies to explore the possibility of a threshold in the dosage range we have mentioned for liver tumors in mice. One variable that certainly ought to be explored in connection with the liver tumors is the induction of microsomal enzymes, because it has been suggested that this induction is intimately tied to the tumorigenicity of a number of chlorinated hydrocarbons as well as to that of phenobarbital [World Health Organization (WHO), 1979]. In fact, the same World Health Organization expert committee recommended specifically that pyrethrins be tested to see whether they increase the incidence of liver tumors in animals, inasmuch as they are known to induce microsomal enzymes of the liver. The results of the bioassays were mixed, although largely negative (ATSDR, 2003). WHO (2001) concluded that there is little indication that pyrethroids should be considered carcinogenic, proving the point of Dr. Hayes.

In the United States, there is extensive concern among the general population about the safety of chemicals. How much concern would remain if the matter were not continuously inflamed by the media is an open question. Regardless of the source and degree of concern, toxicologists would be in a vastly better position to advise if there were more information on the effects of small dosages. The small number of studies that have been carried out simply is not enough. Only a poor experiment does not raise more questions than it answers. Even those who disagree with the interpretation of the ED 01 study in relation to hepatic tumors could hardly argue that something might not be learned by repeating the study and including dosages at and below the predicted intercepts of the logprobit curves with the control levels. It also would be difficult to argue that something might not be learned by comparing the dosages of 2-AAF that induce microsomal enzymes with those that increase the incidence of liver tumors in mice.

The basic reason for wanting to learn more about the effects of small dosages is scientific and, therefore, intellectual. To be sure, any progress that might be made could have important implications for the regulation of chemicals and for our confidence in that regulation. However, no scientific or practical progress can be made by ignoring the statistical results for control animals or by imagining that studies of chemical or physical carcinogens provide any information about the cause of neoplasia among the controls. In short, no progress can be made by those who attempt to extrapolate from animal experiments to humans without obtaining more information than can be measured in the animals.

There is an analogy-and a contrast-between the concept of the cumulative lognormal curve in toxicology and Einstein's proposal for the equivalence of energy and matter ($E = mc^2$). Both concepts were based on theoretical considerations with little or no support from common sense and both were unproved when first proposed. That is where the analogy ends. Physicists have sought every possible way of testing the validity of the equation $E = mc^2$. They have carried out meticulous experiments, and they have gradually accumulated evidence that has made the equation a cornerstone of modern physics. Toxicologists, on the other hand, have done so little to prove or disprove the theory of the lognormal response of organisms to toxicants that most of the work and the theory have been reviewed in two brief sections. If we toxicologists had been as thorough and energetic as the physicists, we should not be in the strange position of having a theory with no known exception but with so little critical evidence supporting it that few dare to accept it as true.

Hayes' truly visionary words about the need for a theory of toxicology and the dire consequences of claiming exceptions to it are finally coming to fruition in this revised chapter. As postulated in Section 1.1.3 there are no exceptions to the $c \times t$ concept; there are only incompletely controlled experiments or experiments conducted under less than ideal conditions. It must be emphasized that this can be seen only under worst case exposure conditions (continuous exposure) or when an effect is essentially irreversible during the observation period. Somehow we need to recognize the futility of continuing to conduct toxicological experiments in the traditional way. Otherwise, we are just producing some more of the data that raise more questions than provide answers. It is entirely meaningless to continue examining the myriad of microscopic variables without the guiding constraint of laws of the macroscopic variables, which are the dose (concentration at the site of action), the various timescales and the effect.

One more issue needs clarification for the sake of making this section complete. The principles underlying beneficial and detrimental effects are the same and as such this distinction is highly anthropocentric and not scientific. For example, low doses of many bacteriostatic agents promote bacterial growth (which is undesirable from an anthropocentric view, but desirable from a bacterial point of view), whereas high doses kill the bacteria (which is desirable from the anthropocentric viewpoint, but not so from a bacterial

point of view). Another example is the issue of contraceptives. For a couple not wishing to have children, oral contraception is a beneficial effect (desirable). For another couple, yearning for children without fulfillment, the presence of naturally occurring contraceptives in the diet would be an adverse effect (undesirable). Toxicology would be better served and would remain a more credible science if such value-laden terms were avoided. An effect is a dose- and time-dependent action of a chemical on an organism characterized by one or more dose- and time-dependent responses. Low-dose effects are most often beneficial to an organism whereas high-dose effects are for the most part adverse (toxic) effects. We need to remember though that selective toxicity (e.g., antibiotics, pesticides, cancer therapy) can be highly desirable, because of the perceived benefit.

1.3.7.5 Geometric Mean

Francis Galton (1879) pointed out that, in many vital phenomena, equal intervals of effect are produced by logarithmic intervals of stimulus. He used as a specific example Fechner's law, which in its simplest form states that sensation is proportional to the log of stimulus. Galton emphasized that, for such phenomena, the true mean is the geometric one. In the geometric series 1, 2, 4, 8, 16, 32, .., the geometric mean of 4 and 16 is 8 (i.e., 4 : 8 = 8 : 16) and not 10 (i.e., not (4 + l6)/2). Use of the geometric mean where appropriate avoids the consequences of assuming that errors in excess or in deficiency of the truth are equally probable. To show how absurd or misleading this assumption can be, Galton recalled that, because there are giants more than twice as tall as the mean height of their race, the assumption "implies the possibility of the existence of dwarfs whose stature is less than nothing at all."

In his brief rational paper, Galton introduced a more technical mathematical study by Donald McAlister (1879) entitled "The law of the geometric mean." This law has not received the attention or use it deserves. It is appropriate for calculating the average storage of a compound in a population or the average time of death in a series of animals all dosed in the same way. On the other hand, few of the published arithmetic means are so much in error that they ought to be discarded. As Galton (1879) noted, the difference between the arithmetic and the geometric mean is small if the range of the values averaged is narrow.

1.3.7.6 Reproducibility of Results

Ideally, the results of any particular measurement ought to be reproducible in the same laboratory or from one laboratory to another. This becomes especially important when the numerical results may be used as guides for diagnosis and therapy, or when any results may be used to determine whether a compound does or does not satisfy legal criteria (e.g., criteria of registration or residue tolerances). However, results can be meaningful and important even when it is impossible to standardize the conditions to the point that control values are statistically identical from one trial to another.

An astonishing proportion of biological and biochemical studies are recognized as valuable contributions if the results for each experimental group show a clear-cut relation to the results for the corresponding control in the same experiment. Of course, no study can be considered confirmed until the relationships demonstrated in the initial experiment have been redemonstrated in the same laboratory or, even better, in different laboratories.

Whereas all scientific procedures are examined from the standpoint of reproducibility within an experiment, only a few toxicological methods have been examined thoroughly for reproducibility in a broader sense. In these studies, it seldom has been possible to identify all of the causes of variation. In animal experiments, some of the variables discussed in Section 1.4 may be detected (Weil and Wright, 1967).

Probably the most important single factor in determining reproducibility is the objectivity of the end point. In a study of the oral LD 50, for which the end point is clear-cut, different protocols in use in well-established laboratories produced results that differed so little that choice of one or the other would not change the interpretation of the relative hazard of any particular compound. Specifically, the highest and lowest LD 50 values for each of 10 compounds as determined in eight laboratories by various protocols differed by factors ranging from 1.30 to 5.48. The degree of variation was less, but not statistically less, when each laboratory used a reference protocol and a reference stock of rats as compared with (a) reference protocol and rats commonly used in the laboratory or (b) both protocol and rats commonly used in the laboratory (Weil and Wright, 1967).

Far greater differences were found in a study of intralaboratory and interlaboratory variability in the results of eye and skin irritation tests, for which the end points are subjective. Although other factors were involved, it was concluded that the main factor contributing to variability was difficulty in reading the reactions. Although numerical factors of difference (between highest and lowest values) could not be assigned, some of the differences obviously were very great. The majority of laboratories performed the tests competently and reproducibly; however, others were far afield. Some materials were rated the most irritating by some laboratories and rated the least irritating by others. Some of the laboratories that were most out of line were industrial and some were governmental. Therefore, restricting testing to any one type of laboratory would not solve the problem. In fact, it was concluded that the tests that had been in general use for 20 years were no longer dependable ways of classifying a material as an irritant or a nonirritant. It was suggested that modification of the tests themselves would not be helpful but

that careful reeducation of those who perform the tests would be required if any improvement were to be made (Weil and Scala, 1971). The Council of the Society of Toxicology supported this emphasis on training and a lack of emphasis on rigid standardization of protocols (Hayes *et al*., 1971).

One factor that may contribute to the failure of a laboratory to agree with the majority of others in a particular test is unfamiliarity with the test. In a study of the reproducibility of measurements of blood lead, it was noted that some of the laboratories ordinarily had occasion to use the test only a few times per year (Keppler *et al*., 1970). Here is not only one explanation for poor performance but an indication that the study may not have reflected the accuracy of experienced laboratories. Reeducation would be most efficient if it could be provided when needed, that is, just before an infrequently used test is required. However, if only a few tests are to be run, it probably would be more efficient to refer them to another laboratory than to arrange training.

1.3.7.7 Abnormal Values in Control Groups

It sometimes occurs that a statistically significant difference between an experimental group and its control depends on an abnormality of the control and not on any deviation in the experimental group. This is an important reason changes, to be indicative of a deleterious effect, must be produced that are dosage-related and illustrate a trend away from the norm for the population under study (Task Force of Past Presidents, 1982; Weil *et al*., 1969).

1.4 DOSAGE-RESPONSE RELATIONSHIPS IN DIFFERENT KINDS OF TOXICITY OR CHANGE

1.4.1 Toxicity (*Sensu Stricto*)

All people with toxicological or medical training are aware that toxicity in the restricted sense corresponds to dosage for any particular compound. However, use of the various procedures described in Section 1.2 for measuring dosage-response relationships is restricted all too often to this limited kind of toxicity.

Toxicity in both the strict and broad sense consists of illness or death. It sometimes is implied, without toxicological or logical basis, that specifying the kind of illness involves some change in underlying principle over and above the restrictions imposed by the specification. Neurotoxicity is restricted to the nervous system and teratology to the embryonic stages, but the broad principles of toxicology remain unchanged.

The less common or less familiar a phenomenon is, the more likely that its relationship to an actual or supposed etiology will be viewed qualitatively rather than quantitatively. Phenomena often viewed in this way include neurotoxicity, teratogenesis, carcinogenesis, mutagenesis, hypersensitivity, and storage, as well as adaptive response of microsomal enzymes.

1.4.2 Neurotoxicity

Neurotoxicity is the delayed but persistent paralysis (polyneuropathy) caused by certain organic phosphorus compounds as well as a few other toxicants, deficiency diseases, and infections. The classical example is "jake leg" paralysis caused by triorthocresyl phosphate (Smith *et al*., 1930). Most studies of neurotoxicity associated with organic phosphorus compounds have attempted to learn which molecular configurations are capable of producing the phenomenon and which are not. When active compounds were investigated qualitatively, it was found that a sufficiently low dosage was tolerated, and progressively larger dosages increased the frequency and severity, and often reduced the latency of neurotoxicity (Aldridge and Barnes, 1961; Cavanagh *et al*., 1961; Davies *et al*., 1960; Siegel *et al*., 1965).

Hayes (1991) points out the time-dependence of neurotoxicity, but also that clear-cut quantitative relationships have not been found, perhaps because they have not been sought. Part of the problem why such studies have not been conducted is a lack of conceptual framework for the experimental design in combination with formidable methodological difficulties. Neurotoxicity is most often irreversible to some extent, although adaptation to neuronal damage is possible as well as repair of the injury in some instances. Thus, in many instances when the underlying dynamic processes are slower than the kinetics (elimination) of the causative agent there will be two or more rate-limiting steps in the recovery process from an insult. Whichever will be the rate-determining step could be elucidated by a careful time course study of the recovery and curve stripping to find the respective half-lives of adaptation, repair, and reversibility. Such experiments have not been conducted to our knowledge. Other types of neurotoxicity, such as loss of the righting reflex or unintentional anesthesia (which are highly reversible), have (a) kinetic process(es) as the rate-determining (-limiting) step(s) in their action. Under conditions of continuous exposure (inhalation) such effects have been often shown to obey Haber's rule of $c \times t = k$ (Flury and Wirth, 1934).

1.4.3 Teratogenesis

Although most research in teratogenesis has been centered on the nature of the phenomenon itself as well as the biological factors, which influence it, a few quantitative studies have been made. These studies illustrate that for any given compound and experimental situation there is a dependable relationship between dosage and effect

(Murphy, 1965; Wilson, 1964). It has been pointed out that there is no way to exclude that any given compound may be teratogenic to some species under certain conditions (Bough *et al*., 1963; Karnofsky, 1965).

Steep dosage-response curves for teratogenic action such as those shown in Fig. 1.14 are not uncommon and, in fact, appear to be the rule. Agents can be tolerated in low dosage without any recognizable effect on development or viability, but most of them that have detectable teratogenicity rather quickly become lethal to all embryos at higher dosages. Between these ranges of normality and lethality, there exists a narrow zone of dosages in which variable numbers of embryos survive with varying degrees of teratogenic involvement. A sharp rise of the dosage-response curve is also characteristic of the teratogenic action of X-radiation (Wilson, 1964).

From the theoretical point of view, the critical timescale in teratogenesis is that of organogenesis, which is such a narrow window in time that it will be difficult if not impossible to determine time-dependence of a teratogenic effect (within that window) experimentally. However, teratogenicity occurs at dynamic steady state because no recovery from it is possible after the narrow window in time passes. In agreement with previous considerations this should yield a very steep dose response because once again nature provides nearly "ideal conditions" for teratogenic experiments. Indeed, Fig. 1.14 demonstrates that the dose-responses for diphenylhydantoininduced orofacial and skeletal anomalies occur with a slope of less than 2. The importance of both kinetic and dynamic considerations in the manifestation of teratogenicity of retinoic acid agonists has been highlighted in Arafa *et al*. (2000).

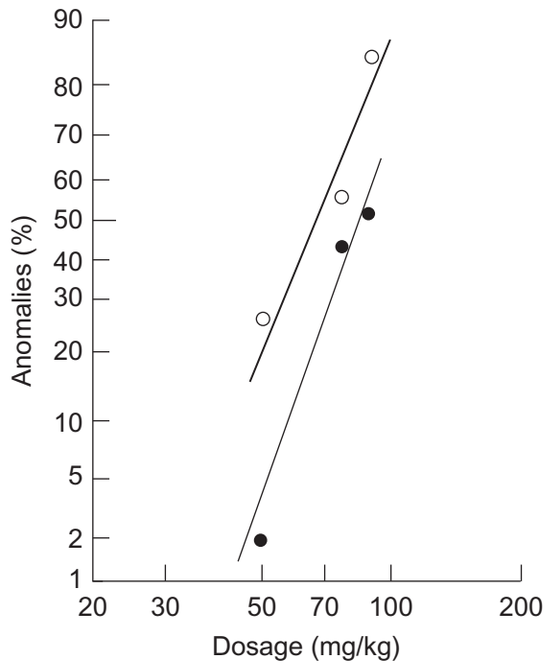

FIGURE 1.14 Incidence of orofacial anomalies (○) and skeletal anomalies (●) in fetal mice whose mothers received different dosages of diphenylhydantoin by intraperitoneal injection on gestation days 11, 12, and 13. Data from Harbison and Becker (1970).

1.4.4 Carcinogenesis

Strong carcinogens demonstrate a striking dosage-response relationship whether expressed on the usual basis of incidence versus dosage (Figs. 1.13 and 1.15) or on the basis of logtime versus logdosage (Fig. 1.11). Thorough reviews of the matter have been written by Druckrey (1967) and by Shabad (1971). In many instances the maximal tolerated dosage of weaker carcinogens is required to reveal carcinogenesis with statistical dependability. From a data base of 52 studies, Haseman (1985) tabulated examples from which he concluded that over two-thirds of the positive results would have been missed if only half the maximal tolerated dosages of the different compounds had been studied. However, so much research on carcinogenesis has been centered on the phenomenon itself and so little attention has been given to quantitation of the actions of chemicals that even some experts in the field seem unfamiliar with the fact that chemical carcinogenesis follows clear-cut dosage-response relationships.

There is no doubt that only a small dosage of certain compounds is necessary to increase the incidence of cancer in susceptible animals. For example, as shown in Table 1.5, the ED 50 for carcinogenesis following a single subcutaneous injection of three of the classical laboratory carcinogens in mice ranges from 0.76 to 4.6 mg/kg. The naturally occurring carcinogen aflatoxin also is effective when administered subcutaneously at a total dosage of about 1.8 mg/kg (Dickens

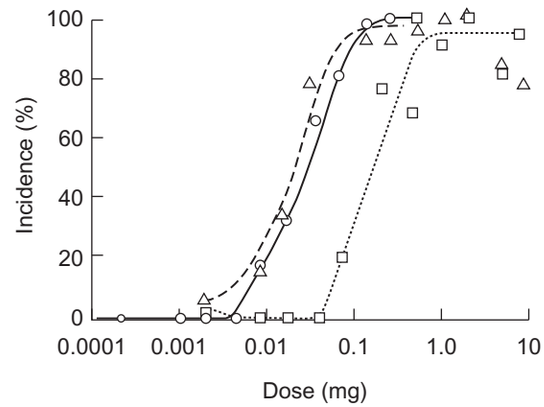

FIGURE 1.15 Responses of mice to graded single doses of each of three polycyclic aromatic hydrocarbos carcinogens dissolved in tricaprylin and injected subcutaneously: methylcholanthrene (○); dibenzanthracene (△); 3,4-benzo[*a*]pyrene (□). Approximately 20 animals per dose of each compound. From National Academy of Sciences (1960) by permission of the Academy.

TABLE 1.5 Toxicity of One Dose of Selected Materials

Material (route)[a]	Species	Sex	Dosage (mg/kg)	Effect	Reference
Botulinal toxin A					
α-fraction (iv)	Mouse	—	0.000,000,27	LD 50	Dasgupta *et al.* (1966)
Unfractionated (ip)	Mouse	—	0.000,001,4	LD 50	Lamanna and Carr (1967)
Same (po)	Mouse	—	0.001,4	LD 50	Lamanna and Carr (1967)
Same (po)	Human	—	<0.000,014	LD 50	Schantz and Sugiyama (1974)
O-ethylmethyl-*S*-phosphorylthiocholine iodide (ip)	Human	—	0.03	LD 50	Holmstedt (1959)
N, N′-Di-n-butylphosphorodismine fluoride (im)	Chicken	F	2.0	LD 50	Davies *et al.* (1966)
Same (im)	Chicken	F	0.05	para[b]	Davies *et al.* (1966)
Dibenzanthracene (sc)	Mouse	M	0.76	ED 50[c]	Bryan and Shimkin (1943)
Methylcholanthrene (sc)	Mouse	M	0.96	ED 50[c]	Bryan and Shimkin (1943)
Benzo[*a*]pyrene (sc)	Mouse	M	4.6	ED 50[c]	Bryan and Shimkin (1943)
Aldicarb	Rat	M	0.8	LD 50	Gaines (1969)
Tetraethylpyrophosphate	Rat	M	0.8	LD 50	Gaines (1969)
Parathion	Rat	M	13	LD 50	Gaines (1960)
Endrin	Rat	M	17.8	LD 50	Gaines (1960)
Arsenic trioxide	Rat	M	72	LD 50	Gaines (1968)[d]
Nicotine sulfate	Rat	F	83	LD 50	Gaines (1960)
DDT	Rat	M	113	LD 50	Gaines (1960)
Pyrethrum	Rat	M	470	LD 50	Gaines (1968)[d]
Acetylsalicylic acid	Rat	—	1360	LD 50	Eagle and Carlson (1950)
Malathion	Rat	M	1375	LD 50	Gaines (1960)
Sodium chloride	Rat	M	3550	LD 50	Gaines (1968)[d]
Difenphos	Rat	M	8600	LD 50	Gaines *et al.* (1967)

[a]*Doses are oral unless otherwise indicated; iv, intravenous; ip, intraperitoneal; po, per os; im, intramuscular; sc, subcutaneous.*
[b]*Para, paralysis.*
[c]*Carcinogenesis.*
[d]*T. B. Gaines; personal communication to W. J. Hayes, Jr. (1968).*

et al., 1966), but its danger by the oral route is more important. Dietary intake of aflatoxin B1 for only 2 weeks at a total dosage of about 2.6 mg/kg produces carcinoma in male rats and a lower daily intake for a longer period also produces cancer when the total dosage is less than 0.5 mg/kg (Wogan and Newberne, 1967).

Unfortunately, little or no attention has been given to the dosage-response relationships of weak carcinogens.

Problems of the quantitative study of the effects of small dosages discussed in Section 1.3.7.4 are relevant to carcinogens as well as other toxicants. In fact, Fig. 1.13 is based on a study of 20-methylcholanthrene, and Fig. 1.15 is based on a study of three carcinogens.

There is evidence, sometimes of a very tenuous nature, that some pesticides are tumorigens if not carcinogens.

Hayes (1991) recognized that carcinogenesis is one of the most distorted issues of science mainly because of the enormous societal concern about cancer, which has become one of the major causes of old-age-related death. Physicians have been cognizant of the capability of chemical and physical agents to induce cancer at least since Sir Parcifal Pott's observation of scrotal cancer in chimney sweeps. This observation was confirmed experimentally in controlled studies in the early 20th century (Yamagawa and Ichikawa, 1915). Therefore, chemical-induced cancer was considered early on as one end point of toxicity. Initially,

potent carcinogens were studied for which the carcinogenic dose-responses were to the left of nonspecific toxicity and/or old-age-related death. Therefore, complete and steep dose-responses were obtained as shown by Fig. 1.15. The slopes of these dose-responses are somewhat distorted, because of plotting dose rate (daily dose) instead of dose (cumulative dose) vs. effect. When plotted on the appropriate dose scale the carcinogenic dose responses are as steep as any other dose-responses discussed thus far (Rozman *et al*., 1996). When toxicologists started studying less potent carcinogens the dose-responses became truncated by the 2-year terminal sacrifice or the natural life span of the experimental animals. Introduction of the MTD reduced carcinogenesis to a largely qualitative yes-or-no-type phenomenon determined by a statistical comparison of treated animals to controls. The fact that at MTD/2 about 50% of the chemicals have shown no statistical difference to controls indicates, in agreement with theory, that most of the recently conducted bioassays targeted weak to very weak carcinogens for which the carcinogenic dose response coincides with the dose-response of nonspecific toxicity. As is the case with other end points of toxicity, there are no shallow cancer dose-responses, there are truncated cancer dose-responses, incorrectly plotted cancer dose-responses, and there are incompletely controlled cancer studies measuring at the same time toxicity and recovery when the kinetic or dynamic half-life of a carcinogen is very short and dosing occurs once or twice (feeding) a day. It is unfortunate that toxicologists surrendered their knowledge and understanding of toxic phenomena to the modelers and to statistics, which describe that which is unknown or unknowable. The price paid has been a stagnation of the development of the theory of toxicology.

1.4.5 Mutagenesis

Nearly all studies of chemical mutagenesis have been concerned with identifying mechanisms of action or with learning whether selected chemicals can or cannot cause some mutagenic effect in the system under study. Little attention has been paid to dosage-response relationships. However, when such a relationship is sought it has always been found. Examples from his own work and that of others on the induction of phage were given by Heinemann (1971). Dominant lethal mutations produced in insects and mammals by many alkylating agents and some other compounds regularly show dosage-response relationships. The toxicity of some of these compounds considered for use as insect chemosterilants has been reviewed (Hayes, 1964, 1968). Mutagenicity is one of the key lessons of modern toxicology. Early on, it was hailed as an inexpensive way to predict carcinogenicity. Later, the claim was reduced to at least predicting DNA-reactivity. Neither hope was fulfilled and this widely used assay is on its way to the role of an ignominious prediction never materialized.

1.4.6 Hypersensitivity and Allergy

The extreme sensitivity of some people to certain substances is illustrated by the fact that anaphylactic reactions to penicillin have been produced during skin testing with as little as 10 units of the drug (Mayer *et al*., 1953).

The fact that some people are allergic and others are either not allergic or even highly resistant tends to obscure the fact that the various forms of hypersensitivity are dosage-related within a homogeneous population. Individuals who suffer from allergy often find that a reduction of dosage will lead to clinical improvement. For example, people who are sensitive to pollen often get some relief by closing most of the air inlets of their homes and placing filters on the remaining ones, even though this procedure does not eliminate their exposure to pollen but merely reduces it. Under experimental conditions in which the susceptibility of animals was made uniform by passive transfer, the onset of anaphylaxis was directly related to the doses of antigen (Pruzansky *et al*., 1959). Human leukocytes isolated from ragweed-sensitive donors release histamine at rates determined by the concentrations of purified antigen derived from the pollen (Lichtenstein and Osler, 1964). Other forms of hypersensitivity may be dosage-related also. For example, blood dyscrasias, especially aplastic anemia, are a recognized hazard of the otherwise valuable drug chloramphenicol. Hodgkinson (1954) showed that these dangerous side effects occurred predominately in cases in which the drug had been administered at a rate significantly higher than usual.

The fact that even hypersensitivity is often dosage-related emphasizes the importance of searching for suspected but undemonstrated dangers of a particular compound among people whose exposure is most intensive and prolonged.

Hypersensitivity and allergy are special cases of toxic responses, which occur in subjects that are not part of the normal distribution in terms of this particular and possibly of some other responses. Expressed differently, once an individual has been sensitized, it is no longer the same subject as before. Rather, the sensitized individual belongs to the normal distribution of a sensitized population with its own dose- and time-responses. It will be very difficult to sort out dose- and time-responses in such individuals, because the sensitization occurs in an individual who is still part of the normal distribution of the general population while being sensitized but becomes part of another normal distribution thereafter.

1.4.7 Induction of Enzymes

Microsomal enzymes, offer some explanation for a number of otherwise obscure facts in toxicology. It is generally admitted that the net effect of these enzymes is adaptive for the organisms, but it has been suggested that stimulation of enzymes by one chemical will lead to greater injury to the organism when faced with some other challenge. Regardless

of the final toxicological evaluation of the process of induction, it is clear from work already completed that there are orderly relationships between dosage and response for compounds that stimulate microsomal enzymes. In fact, one of the very early papers by Conney *et al*. (1956) demonstrated very clear dosage-response relationships for demethylase and DAB-reductase following injection of 3-methylcholanthrene. The same paper demonstrated a similarly clear relationship for inhibition by ethionine.

Apparently the first such studies of pesticides as inducers of microsomal enzymes were those of Hart and his colleagues regarding chlordane and DDT. No obvious dosage-response relationships were found with either single or multiple doses of chlordane (Hart and Fouts, 1963). Some indication of a dosage- response relationship was evidence from tabular values for DDT, but the relationship apparently was not discussed (Hart and Fouts, 1963) or discussed only briefly (Hart and Fouts, 1965) in connection with these early studies. Kinoshita *et al*. (1966) first demonstrated clearly a dosage-related effect of DDT and toxaphene on enzyme induction. The result has been confirmed in connection with DDT (Gillett, 1968; Hoffman *et al*., 1970) and other compounds (Gielen and Nebert, 1971; Hoffman *et al*., 1968).

Sufficiently small dosages produce no detectable effect on enzyme activity. There is considerable evidence that the threshold dosage for enzyme induction corresponds to the upper limit of intake that can be metabolized by the unstimulated liver (Hoffman *et al*., 1970).

The threshold dosage of DDT for induction of various microsomal enzymes in the rat has been estimated at about 0.05 mg/kg/day (i.e., a dietary level of 1 ppm) (Kinoshita *et al*., 1966) or 0.5 mg/kg/day (Schwabe and Wendling, 1967). Datta and Nelson (1968) found that a dietary level of 4 ppm (about 0.2 mg/kg/day) induced enzymes. Gillett (1968) found the threshold to be 0.125 mg/kg/day. Street *et al*. (1969) estimated the threshold at 0.05 mg/kg/day. The different estimates are not necessarily inconsistent, because they depend on different test systems. In any event, the lowest estimate (0.05 mg/kg/day) is only 0.2 times that known to be effective in humans (Laws *et al*., 1967; Poland *et al*., 1970) whereas it is 50 times greater than the average dietary intake of all DDT-related materials by a 16- to 19-year-old man during the mid l960s, that is, 0.0009 mg/kg/day (Duggan, 1968).

The enzyme-inducing dosage of DDT (0.5 mg/kg/day) used by Schwabe and Wendling (1967) led in 14 days to a storage level of 10 ppm in the adipose tissue of rats. The dosage of 0.2 mg/kg/day used by Datta and Nelson produced in 20 weeks a storage of 39 and 76 ppm in the adipose tissue of male and female rats, respectively. Twelve weeks after dietary feeding of DDT was stopped, the storage levels of DDT-related materials had fallen to 11 and 21 ppm in males and females, respectively, compared with 6 and 9 ppm, respectively, in the controls. The rats previously fed DDT still showed some induction of liver enzymes 12 weeks after dosing was stopped. Neither the rats described by Schwabe and Wendling (1967) nor those described by Datta and Nelson showed a steady state of DDT storage when their values were between 10 and 21 ppm. It is, therefore, open to serious question whether these storage values are at all comparable with those found in people in the general population.

The order of Sections 1.4.8 and 1.4.7 has been reversed in this edition also, because enzyme induction is part of the dynamics of a chemical (what does the chemical do to the organism?) and as such belongs to the same category as neurotoxicity, cancer, etc. Metabolism and storage, on the other hand, are part of the kinetics of a chemical (What does the organism do to the chemical?).

Enzyme induction is for the most part a transient adaptive response of an organism which promotes biotransformation of the causative agent and thereby contributes together with excretion to detoxification (recovery by kinetics). In rare instances, enzyme induction will lead to metabolic activation, namely, making the chemical itself or other compounds more toxic to the host (Parkinson, 1996).

Enzyme induction is a highly reversible phenomenon and if the half-life of the causative agent is short, there will be few if any adverse consequences to the host unless exposure to the chemical is continuous. The other limiting condition is represented by chemicals having very long kinetic half-lives, in which case even after a 90-day off-dose period there is virtually no reversibility of induction observable (Viluksela *et al*., 1997). This is the kinetic equivalent of an essentially permanently altered organism as discussed for hypersensitivity, which in contrast to this has dynamic causes. As long as the induction persists, the individual belongs to a different population in terms of normal distribution. Thus, he or she may be more or less sensitive to other toxic agents depending on whether metabolic activation or deactivation (detoxification) is the rate-determining step. For example, induction of enzymes metabolizing acetaminophen to its toxic metabolite aggravates its hepatotoxicity, but enzyme-inducing doses of TCDD reduce mammary tumors highly significantly below controls (Rozman *et al*., 1993, 1996; Rozman *et al*., 2005). Enzyme induction *per se* is not a toxic effect; it is just an effect. It depends on the consequences whether or not enzyme induction will lead to decreased or increased toxicity or remains inconsequential for the host. A lack of conceptualization has resulted in the loss of a great many potentially superior drugs.

1.4.8 Metabolism and Storage

The relationship between equilibrium storage and daily dosage for DDT in the human, rat, rhesus monkey, dog, and turkey are shown in Fig 1.16. It is clear that equilibrium storage corresponds to daily dosage in all species studied. However, the details of this relationship differ according to species and, at least in the rat, according to sex and dosage level. Specifically, storage is the same in male and female rats up

to a dosage of about 0.02 mg/kg/day, but above this level storage is greater in females. This either represents an experimental artifact or some other error, because male rats must have lower storage levels than females, because of greater growth delution (faster growth). Although species differences are to be expected, the pattern reported for the dog is remarkably different from the patterns for other species and ought to be reexplored, especially at lower dosage levels.

In humans, some elements and compounds are stored in progressively greater concentration with increasing age. This requires further study to determine the cause in each instance; it is not a reason to doubt the general principle of equilibrium. Possible causes include *(a)* excretion so slow that equilibrium is not achieved during the interval involved, *(b)* a combination of very slow excretion and decreasing dosage so that storage in older people still reflects their higher dosage before the younger people were born, and *(c)* progressive decline in the ability to metabolize or excrete the material based either on a specific injury by the toxicant (as in the case of radium) or on age *per se.*

Storage is part of the kinetic phenomenon of distribution, and metabolism is part of the kinetic process of elimination; and as such both are distinctly different from the dynamic part of the decision tree (Section 1.1.1) represented by all other subsections of Section 1.4.

Storage is due to the fact that one or several body compartments with slow blood perfusion have high affinity for a given chemical, which leads to its redistribution provided that its distribution into these compartments is faster than its elimination. Thus, the timescale of redistribution depends on the ratio of the distribution and elimination half-lives of a chemical. For example, the initial distribution of lead results in very high liver concentrations. The very slow excretion of lead allows for virtually complete redistribution into bone, which is a compartment extremely slow to equilibrate with the central compartment. If the half-life of lead could be decreased to a few minutes, there would be little storage in or redistribution into the bone matrix at all.

Data of Fig. 1.16 indicate a linear relationship between dose rate and storage in adipose tissue of DDT in different species, whereby the slopes are apparently species-dependent. There is no doubt that during the early phase of a subchronic-chronic study there will be linear accumulation of a compound that has high affinity for a tissue combined with slow excretion. DDT preferentially accumulates in fat (Fig. 1.16), whereas TCDD accumulates to about the same extent in adipose tissue as in liver (Weber *et al*., 1993). However, after 3.32 distribution half-lives 90% and after 6.64 half-lives 99% of maximum storage will be reached for either compound, implying lack of linearity in the storage of chemicals beyond the initial stage. Different slopes in different species and genders are related to different body composition, differential metabolism, and vastly different growth rates of the species or gender discussed, which – if studied carefully – would account for all the differences.

Metabolism or biotransformation of chemicals is clearly an important aspect of their kinetics, but the role of biotransformation in toxicology is overrated. Biotransformation

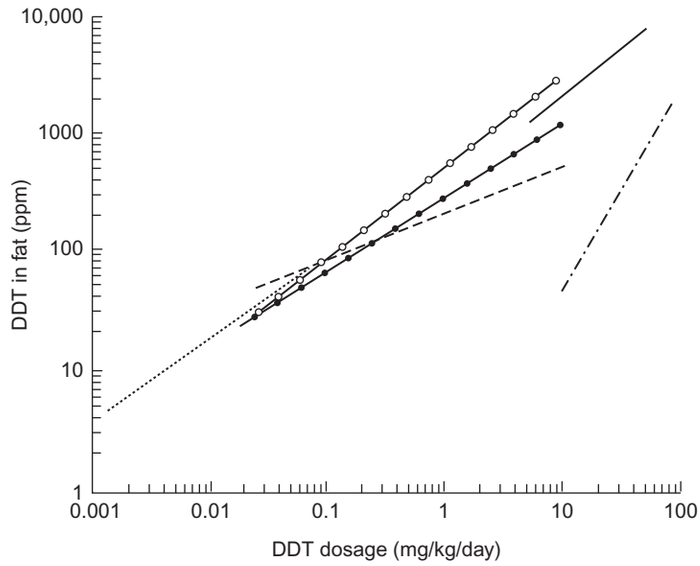

FIGURE 1.16 Storage of DDT in the adipose tissue of human (…), rat (o — o, female; •—•, male), rhesus monkey (—), dog (– · –), and turkey (– – –). The curves have not been extrapolated beyond the dosage levels studied.

amounts to altering a chemical into a different chemical moiety, which is one form of elimination. It is a removal of the metabolite from the shifting equilibrium of the parent compound, resulting in the metabolite taking on its own kinetic/dynamic life. Most metabolites are more water soluble (particularly phase II products except a few acetylated and methylated compounds) than the parent chemical and hence are excreted more rapidly. After chronic administration, their steady state concentration is often negligible compared to the parent compound. This amounts to kinetic recovery from intoxication. If a metabolite formed is of comparable or higher intrinsic toxicity (agonists) than the parent compound then the question of additivity must be entertained, provided that the parent compound and metabolite(s) act by the same mechanism. If they act independently (different mechanism of action) the more toxic one(s) will dominate the manifestation of toxicity. It is only in very rare instances that in such a constellation we have the appearance of a synergistic interaction, which has either kinetic or dynamic reasons or both in combination.

1.5 FACTORS INFLUENCING TOXICITY OF ANY KIND

Although dosage and time are the main factors determining whether or not a particular chemical will produce a given effect, there are other factors that influence response. Factors of a biological nature include route of exposure, species and individual differences, sex, age, nutrition, and disease. Physiochemical factors include temperature and other environmental variables, and of course, the schedule and duration of dosage and the formulation in which the chemical is administered. There is no theoretical and frequently little practical limit to the range of dosages that may be explored experimentally, and there is frequently little practical limit to the range of human dosages that may be encountered at least occasionally. Thus, at one end of the spectrum it may be possible to find unexposed populations and at the other end of the spectrum to find an occasional person who is killed by accidental ingestion of a single large dose. Compared with dosage (and time), the other factors that influence response to a particular chemical have been subject to less quantitative study. However, some have been studied, and it can be said, for example, that pesticides are, on the average, a little over four times more toxic by the oral route than by the dermal route. Factors other than dosage (and time) are important only in special circumstances. Thus, altitude (atmospheric pressure) is unimportant for toxicology in the parts of the world where most people live, but becomes progressively more important in connection with some compounds as altitude increases from 5000 feet and progressively greater strain is placed on cardiorespiratory function. Certain factors are almost universally relevant in toxicology and may affect humans, whereas other factors (such as the details of caging) apply directly to animal experiments only.

The response of liver microsomal enzymes to dosage was mentioned in Section 1.4.7. Differences in these enzymes or in their ability to be induced explain many differences in susceptibility to poisoning associated with interaction of compounds, differences in species, sex, age, and perhaps other factors.

In addition to differences in metabolizing enzymes, changes in membranes, ionization, protein binding, and bile flow in some instances may explain observed interactions of compounds or differences of susceptibility to poisons associated with age, diet, and the like.

Reviews of the effects of one or more factors provide details and references beyond the scope of this chapter; they include articles by Clough (1982), Fortmeyer (1982), Everett (1984), and Rao (1986).

Hayes (1991) identified the four independent variables (compound, subject, dose, and time) of toxicity (effect) and a number of circumstance- or experiment-dependent variables (sex, age, nutrition, disease, temperature, etc.). Of the four fundamental variables of toxicity, compound and subject are implicit variables, because in the absence of one, the other, or both, there is no toxicity. Didactically it would be more advantageous to keep compound and subject (Sections 1.5.2, 1.5.3, 1.5.7, and 1.5.8, 1.5.9, 1.5.10, etc.) together because they determine the qualitative aspects of toxicity (potential spectrum of effects), whereas dose and time provide the quantitative framework for toxicity (Sections 1.5.1, 1.5.4, 1.5.5, and 1.5.6). It was decided, however, to leave the sequence of sections and subsections intact until perhaps a later edition, to retain the historical perspective, because rearranging the sequence would not alter the issues involved.

1.5.1 Dosage

Control of dosage is the basis of almost all safety assessment in the use of chemicals. This rule applies not only to compounds of relatively high toxicity, but also to compounds of low toxicity, including those necessary to life. Babies have been killed by putting salt in their formula in place of sugar (Finberg *et al*., 1963), and it is said that the ancient Chinese carried out executions using water as a toxicant. On the other hand, all of us tolerate traces of arsenic, lead, and mercury (Monier-Williams, 1949), which are naturally occurring elements widely distributed in food and water. They are found in marine fish and in undeveloped areas where they have no use in industry or as pesticides.

A sufficiently large dosage of an ordinarily harmless material is fatal. On the other hand, a sufficiently small dosage of the most virulent poison is without effect. For every compound, dosage can make the difference between health and death; in this sense the importance of this factor is infinite (see clarification by Rozman and Doull below).

Although age, nutrition, and perhaps other factors may be independent determinants of toxicity in animals of the same strain and sex (Sections 1.5.10 and 1.5.11), it is astonishing how infrequently the effective dosage for small and large (mainly juvenile and mature) animals of the same strain can be distinguished statistically. This conclusion is consistent with the results of a study of botulinum toxin (Lamanna and Hart, 1968), which was certainly the most thorough investigation of the relationship between body size and effective dosage. Even though the extreme affinity of botulinum toxin for its receptor is unique, it still follows that strain and species differences, which involve many compounds and often are substantial, cannot be explained by differences of size *per se.* Similarly the striking difference of the susceptibility of young and old rats to 1-naphthalenyl thiourea (ANTU) cannot be explained by their size. Dosage-response relationships that are truly different are emphasized, not hidden, when expressed in terms of body weight.

Dose and time are inexorably bound together in the $c \times t$ relationship above the $c \times t$ lifetime threshold if exposure is continuous to manifestation of effect. If all timescales are kept constant then the effect becomes solely dose dependent (see Section 1.1.4). Such "pure" dose responses can be particularly frequently observed in *in vitro* experiments with constant incubation time. When the dose is kept constant and one timescale is allowed to vary, "pure" time responses emerge (see Section 1.1.4). Therefore, "pure" dose-and-time responses are limiting cases of the general constellation when toxicity is both dose-and time-dependent.

The experiments of Lamanna and Hart (1968) were conducted under isoeffective conditions, because LD 50s were compared. For most of the substances tested a linear relationship was found between log(LD 50) and log(body weight). Geyer *et al*. (1990, 1993) also found a linear relationship between log(LD 50) and log(total body fat content) for TCDD among more than 20 species and strains of mammals. They argued that total body fat content was a surrogate measure of time because "fatter" animals intoxicated by TCDD lived longer than their leaner counterparts. It could be argued that, like body fat for lipid-soluble compounds, body weight is a good surrogate of time for water-soluble substances. Therefore, it is likely that the good correlation for all but one of the chemicals investigated by Lamanna and Hart (1968) reflects Haber's rule in its logarithmic form. Of 16 compounds tested, only one appeared to deviate from linearity, which was most likely due to an unidentified variable related to the fuzziness of the end point of measurement. All 15 compounds obeying linearity had short time to death and appeared to cause death by a similar mechanism (neurotoxicity) of action. The one anomaly (ANTU) caused lung edema, the development of which can take longer than one day (limit of the observation period). Therefore, almost certainly supralethal doses must have been used to cause death within one day, which would lead to departure from linearity if deaths due to other causes started occurring concurrently (Section 1.3.3.1). This is a very good example to illustrate what a theory-and the lack thereof-does to a discipline. Lamanna and Hart (1968) in the absence of a theory opted to take a cautious stand and emphasize the lack of generalizability of their finding, because of the presumed exception, and thereby lost the important informational content of their study. Under the guidance of a theory an important generalizable phenomenon is emerging from their data with the understanding that any effect studied under conditions of an unfavorable ratio between observation period and time to effect will deviate from linearity.

1.5.2 Compound

1.5.2.1 Primary Compounds

Compounds show a tremendous range of inherent toxicity. Pesticides constitute only a small proportion of all industrial chemicals, but even pesticides show a wide range of toxicity. For example, the oral toxicity of tetraethylpyrophosphate (TEPP) is approximately 588 times greater than that of a pyrethrum extract. However, it must not be supposed that the difference depends on the fact that one of the compounds is synthetic and the other of plant origin, because the difference in toxicity is sometimes reversed. Nicotine, a plant product, is about 103 times more toxic than difenphos, a synthetic organic phosphorus compound. The most toxic materials known are produced by living organisms.

Table 1.5 illustrates the range of toxicity produced by one or a few doses of selected pesticides and some other materials. There is some tendency for compounds of similar chemical nature to resemble one another in toxicity (structure/activity relationship). However, the resemblance is more likely to be quantitative than qualitative. Thus, the organic phosphorus compounds all produce a similar clinical picture, but difenphos does so only at a dosage over 10,000 times greater than TEPP. The toxicity of each compound must be judged separately.

Compounds also show variation in inherent toxicity when given repeatedly. Butler (1965) reported that aflatoxin, a poison elaborated in food by certain fungi, produces cancer in rats at a dosage of only 0.01 mg/day, whereas the synthetics dimethylnitrosamine and butter yellow require dosages of 0.75 and 9.0 mg/day, respectively, to produce the same effect.

The fact that some compounds are inherently likely to produce chronic illness whereas others produce acute poisoning only, regardless of the duration of intake, must be reemphasized here.

Again, the theory of toxicology provides an explanation for this widely observed and reported phenomenon which puzzled several generations of toxicologists. What is required though, is the abandonment of semiquantitative notions of time such as acute, subacute, subchronic and

chronic and to replace them with quantitative measurement of time as an independent variable of toxicity. As discussed and explained in section 1.1.4 ideal conditions for studying dose-time-effect relationships are either isoeffective, isotemporal or isodosic. Nature provides many examples when these conditions are met or nearly so. Cyanide is a good example for what is considered an acute poison. The reason for that is that the time to death is short and therefore essentially no recovery occurs between exposure and effect (death). Execution by cyanide in a chamber provided the theoretically ideal condition of steady state exposure. However, in a few instances of nearly fatal cyanide poisoning the individuals recovered with permanent (chronic) brain damage because of transient hypoxia. Asbestos, in contrast, is considered a chronic poison. Asbestos causes cancers (lung cancer and mesothelioma) only after chronic exposure and the dose cannot be increased to levels when acute exposure would cause chronic toxicity (cancer), because it would suffocate the animals acutely. This brief discussion illustrates that long-entreched notions can be outright harmful for the advancement of a discipline because they actively prevent the development of (a) theory(ies), which is a mandatory requirement for any improvement in experimental design.

1.5.2.2 Derived Compounds

Not only do compounds differ in their inherent toxicity, but they differ in the ease with which they undergo chemical change. Some pesticides may decompose during storage. Others change when their residues are exposed to ultraviolet light, plant enzymes, or soil microorganisms. Thus, one or more derivatives, in addition to the original compound, may be absorbed by humans or animals exposed in one of several ways, including exposure by eating food treated earlier by a pesticide. Of course, nearly all compounds (whether viewed as primary or derivative) are metabolized following absorption by humans or animals. No two compounds are exactly alike. Each derivative and metabolite will differ chemically and toxicologically in some degree from its precursor.

There is no rule regarding the relative toxicity of compounds and their nonmetabolic derivatives. Metabolism tends to render compounds more water soluble and less toxic, but there are instances when this is not the case. Peters (1952) coined the term "lethal synthesis" in 1951 for biotransformation of a compound to a significantly more toxic product, which in modern terminology is called metabolic activation.

Full understanding of the toxicology of each pesticide can be acquired only through recognition and study of its derivatives as well as the primary compound. Such study may reveal that the toxicity of a compound depends on a lethal synthesis. This discovery may or may not suggest the possibility of some preventive or therapeutic measure. However, in no event will discovery of the details change the inherent toxicity of the primary compound. Parathion is no more or less toxic since the discovery that its toxicity depends largely on its conversion to paraoxon.

The usual presence of impurities (from synthesis or decomposition) combined with metabolic conversions occurring in an organism turns practically even the purest compound into a more or less complex mixture.

A chemical moiety is one of two essential elements of a toxic interaction, the other one being a subject or a population of subjects. Toxic potency is an intrinsic property of each and all chemicals. It may be defined as the dose of a compound to cause a defined level of toxicity (ED 20, ED 50, or ED 80) at constant time (all timescales must be kept constant) for time points between the minimum lag period of an effect and the maximum life span of a species. Because determination of relative potency at the minimum lag period or maximum life span would require huge populations of experimental animals, it can be determined more conveniently at ED 20 to ED 80 under conditions of toxicokinetic-toxicodynamic steady state. As discussed for Fig. 1.5, toxic potency is not dose dependent unless the experiment is not measuring toxicity, but various ratios of toxicity/recovery. Dose-response curves are certainly always parallel for chemicals acting by the same mechanism (Stahl *et al*., 1992) under conditions of kinetic or dynamic steady state or as long as departure from that condition is minimal during the observation period. This is the only situation when valid structure-activity (relative potency) relationships can be established.

Metabolites Biotransformation usually leads to more water-soluble (phase I) or very water-soluble (phase II) derivatives, which by definition have shorter half-lives than the parent compound. Therefore, in a chronic dosing experiment the steady state concentration of the metabolite will not correspond to the percentage of metabolite formed but a fraction thereof depending on how much shorter the half-life of a metabolite is compared to the parent compound. Thus, even if a phase I metabolite has agonistic properties leading to additivity of effect, it is seldom of practical importance. In addition, phase I biotransformation products are usually rapidly converted to conjugates, which usually do not have agonistic properties with the parent compound and even shorter half-lives. These theoretical considerations are in agreement with the practical experience that biotransformation of chemicals most often represents detoxification (kinetic recovery). The only exception is metabolic activation or lethal synthesis as coined by Peters (1952) and cited by Hayes (1991), which is extremely well-understood compared to the frequency of its occurrence in toxicology. The questions to be asked in this case are not different in principle, except that now the metabolite rather than the parent compound is the most toxic component. If they act by the same mechanism (agonists in a broader sense than used in pharmacology) then the significance of additivity

depends on the relative kinetics of the two or more compounds. It should be kept in mind though, that if the half-life of a more toxic metabolite is much, much shorter than that of the parent compound, its role in, or contribution to, overall toxicity might still become insignificant due to its correspondingly lower steady state concentration.

Impurities TCDD was an impurity (a few parts per million) in early batches of Agent Orange (2,4,5-T and 2,4-D mixture) and other chlorophenol-related products due to the synthetic process. Because of its high potency and extremely long half-life, it became virtually the only compound of toxicological concern in these products (Kimbrough, 1980). Contrary to this constellation, the presence of small quantities of chlordecone in mirex batches is of little toxicological significance, because its potency is not that much greater than that of mirex and its half-life is shorter (ATSDR, l995a). However, chlordecone is a neurotoxin in its own right and given sufficient exposure will cause this type of toxicity.

1.5.3 Interaction of Compounds

In a broad sense, it is probable that all compounds in the body interact, directly or otherwise. Most of the interactions are so complex, obscure, or trivial that they remain and most of them, should remain unidentified. However, some foreign chemicals have distinct interactions in the body, and in some instances the mechanisms of these interactions have been identified. The compounds that interact may be two or more drugs, may be two or more poisons, or may be an active ingredient(s) and one or more vehicles or other constituents of a formulation sold as a drug or pesticide.

Some examples of interaction not mentioned in the following paragraphs may be found as part of the discussion of individual compounds or groups of compounds in other parts of this book.

1.5.3.1 Kinds of Interaction

The effects of different foreign chemicals may *(a)* mutually interfere with one another, *(b)* be simply additive, or *(c)* potentiate one another. The essentially additive relationship is the most common. Both exceptional conditions-mutual antagonism and potentiation-may be of practical and theoretical importance. From a practical standpoint, interference (antagonism) between two compounds may cancel the benefit or counteract the injury expected from one of them. Potentiation may increase benefit or harm depending on circumstances. From a theoretical standpoint, study of either antagonism or potentiation often leads to a better understanding of the mechanisms of action of the compounds involved.

Methods of Measuring Interaction It has been pointed out that, for statistical reasons, it is possible to estimate the dosage responsible for an ED 50 more accurately than the dosage responsible for some greater or lesser effect. It is for this reason that in studying the interaction of two or more compounds, they are often administered in equal fractions of their respective ED 50 values. If two compounds are compared, the dosages should be a geometric series based on one-half, for three compounds the dosages should be a geometric series based on one-third, and so on. Thus, if the effects of two compounds are exactly additive, administration of half an ED 50 compound A and half an ED 50 compound B should result in exactly one ED 50 for the mixture. This relationship may be written as

$$\frac{1}{2}(ED50_A + ED50_B) \cdot 1.0 = 1ED50_M$$

If the compounds are antagonistic by a factor of 2, the relationship may be written

$$(1ED50_A + 1ED50_B) \cdot 0.5 = 1ED50_M$$

If the effects of the compounds potentiate one another by a factor of 4, the relationship may be written

$$\left(\frac{1}{8}ED50_A + \frac{1}{8}ED50_B\right) \cdot 4.0 = 1ED50_M$$

Written in this way the multiplicand indicates the ratio between the observed and the expected ED 50 for the mixture and expressed the degree and kind of interaction. Thus, 1.0 indicates an exactly additive relationship, progressively smaller fractions indicate progressively greater antagonism, and numbers progressively greater than 1.0 indicate progressively greater potentiation. Actually, the error of measurement is such that fractions or numbers differing from 1.0 by no more than a factor of 2 or 3 cannot be distinguished from the simple additive relationship.

In some instances, it is desirable to study the interaction of two compounds at many dosage ratios. The results of such tests may be recorded in a diagram such as that shown in Fig 1.17. This kind of diagram was introduced in 1926 through a paper devoted to theoretical and mathematical considerations (Loewe and Muischnek, 1926) and another dealing with the antagonism between barbital and aminopyrine (Kaer and Loewe, 1926).

Loewe and Muischnek (1926) introduced the term "isobole" (from the Greek *isos,* equal, and *bolos,* a blow or stroke) to designate a line passing through points of equal action or injury, for example, a series of ED 50 values resulting from administering two compounds in different ratios. In Fig. 1.17, the dotted line indicates all possible comparisons at equal ratios of the two ED 50 values. The three time points on the diagram indicate the same relationships of dosage as those presented by the three equations in the

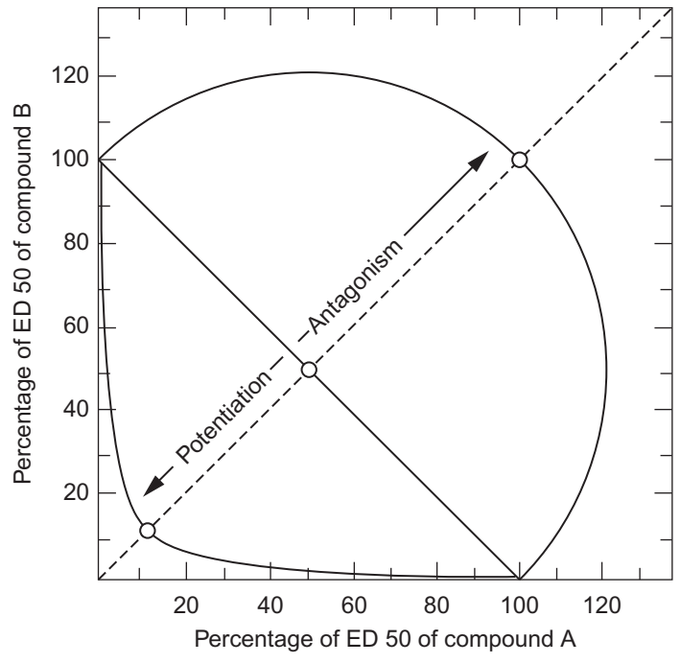

FIGURE 1.17 Isoboles of ED 50 values of compounds A and B illustrating additive, antagonistic, and potentiative interactions. See text for further explanation.

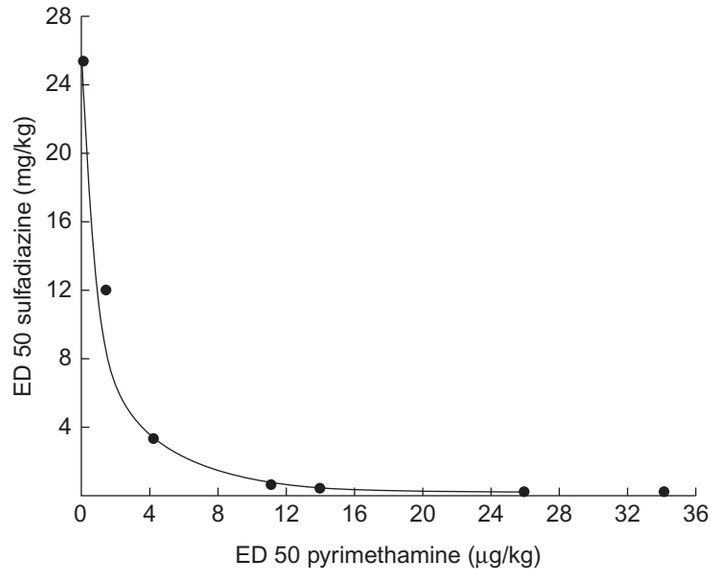

FIGURE 1.18 ED 50s (dosages reducing parasitemia to 50% of the in parasitemia of untreated controls) of pyrimethamine and sulfadiazine, administered both singly and together in various proportions to chicks infected malaria. Each ED 50 was determined graphically from a dosage–response curve. Redrawn from Rollo (1955), by permission of the *British Journal of Pharmacology*.

preceding paragraph. The solid straight line is the isobole of exactly additive action at all dosage ratios. All lines (including one shown) lying to the right and above the isobole represent some degree of potentiation. Real curves are not always symmetrical.

A number of other theoretical relationships or special cases in addition to additive, antagonistic, and potentiative have been mentioned, but whether they exist in nature or can be meaningfully distinguished by the type of diagram shown in Fig. 1.17 is not clear. Examples include a combination

of antagonism and synergism between the same pair of compounds at different dosage ratios, sensitization, and desensitization.

The axes of the diagram need not be measured in percentage, but may indicate dosage directly as shown in Fig. 1.18, which records the toxic (therapeutic) effect of pyrimethamine and sulfadiazine, administered both singly and together in various proportions, to combat malaria organisms infecting chicks.

Systematic Study of Interaction Only a few studies have been made of the possible interactions of compounds in such a way that the results can be compared meaningfully. One such study was that of Keplinger and Deichmann (1967). It involved over 100 combinations of eight chlorinated hydrocarbon insecticides, six organic phosphorous insecticides, and one carbamate insecticide in sets of two and three compounds in rats and mice. The results were expressed as the quotient of the ratio of the expected to observed LD 50 values. The largest quotient obtained was only 2.26, indicating a very small or even questionable potentiation between chlordane and methoxychlor in mice. The smallest quotient obtained was 0.36, indicating a minor degree of antagonism between aldrin and trithion in rats. The data for combinations of three compounds did not reveal any effects of toxicity that could not have been predicted from the combination of two compounds.

In a 2-year rat-feeding study using a combination of six pesticides (DDT, aldrin, pyrethrin, piperonyl butoxide, malathion, and 2,4-D) and eight food additives at use level concentrations or higher, significant alteration of toxicity in comparison with the toxicity of individual substances was not found (Fitzhugh, 1966).

For industrial chemicals as well as for pesticides, the most common joint action is a simple additive one (Smyth *et al*., 1969).

Antagonism Although some instances of interference are encountered in general surveys of interactions, the examples usually show interference that is both small in magnitude and unexplained.

A rapid change in the degree of antagonism may be of more clinical significance than antagonism *per se.* For example, Cucinell *et al*. (1966) reported a fatal hemorrhage in a patient who had received chloral hydrate and bishydroxycoumarin in combination without ill effect. However, when medication with chloral hydrate was stopped but bishydroxycoumarin was continued, the prothrombin time increased and hemorrhage occurred. It was later shown that chloral hydrate stimulates the metabolism of this anticoagulant. The danger lies in too rapid withdrawal of the inducer without an appropriate reduction in the dose of the anticoagulant. The same danger does not exist in connection with inducers that are stored to a significant degree in the tissues because, even if their administration is discontinued, their action decreases very gradually because of their slow elimination from the tissue.

Potentiation A few examples of clinically important potentiation are known to involve pesticides. Many chlorinated hydrocarbon solvents and fumigants, notably carbon tetrachloride, are much more likely to injure the liver if alcohol is consumed at the same time. The hepatotoxicity of many haloalkanes is potentiated by many compounds that induce microsomal enzymes of the liver, by exogenous ketones, including chlordecone, or by metabolic ketosis (Hewitt *et al*., 1980). By a totally different mechanism, the dithiocarbamate fungicides, which are closely related to disulfiram, interfere with the metabolism of alcohol so that alcohol becomes more toxic.

True potentiation is a comparatively rare phenomenon except in connection with certain organic phosphorus insecticides and at least some classes of teratogens. The reason for the interaction of organic phosphorus compounds is that many of them inhibit aliesterases responsible for the efficient detoxification of some other members of the same class (Su *et al*., 1971). This is the mechanism that explains the potentiation of the toxicity of malathion by ethyl p-nitrophenyl thionobenzenephosphonate (EPN) when the dosage of both is substantial (Murphy and DuBois, 1957). However, if the dosage of the two compounds is sufficiently small, there is enough enzyme to detoxify both of them, and the phenomenon of potentiation is not manifest. Rider *et al*. (1959) have shown that people can tolerate 3 mg/day of EPN plus 16 mg/day of malathion or 6 mg/day of EPN plus 8 mg/day of malathion for prolonged periods without significant depression of red cell or plasma cholinesterase. The combination of 6 mg/day of EPN plus 16 mg/day of malathion (Rider *et al*., 1959) or 16 and 5 mg/person/day, respectively (Moeller and Rider, 1962a), did produce asymptomatic depression of both enzymes, but the effect was only additive. No potentiation was noted. The highest dosage of malathion alone tolerated without even slight inhibition of cholinesterase is 16 mg/person/day (Moeller and Rider, 1962a), whereas that for EPN is 6 mg/person/day (Rider *et al*., 1959). Thus, potentiation among this class of compounds may be important for overexposed workers but not for people who ingest residues on foods.

Other mechanisms of interaction are outlined in Section 1.5.3.2. In a study of the interaction of six recognized teratogens, it was found that all pairs showed appreciable potentiation of teratogenic action provided the dosage of each was above a level producing at least a 1 % effect. In several instances, potentiation occurred even when one or more materials were given at sub-threshold dosage. No such consistent pattern of interaction was observed regarding intrauterine death (Wilson, 1964).

It seems likely that the mechanism of cocarcinogenesis will not be explained until neoplasia itself is explained. However, in what may have been the only quantitative

study of this kind of potentiation, a dosage-response relationship was found not only for 9,10-dimethyl- 1,2-benzanthracene but also for this compound in combination with the cocarcinogen croton oil (Graffi, 1953).

Whether potentiation will be of any practical importance depends on its degree and on the chance a person or animal may have simultaneous exposures to adequate amounts of two potentiating compounds. Potentiation as high as four- or five-fold, such as that seen with some organic phosphorus insecticides, is of limited toxicological importance. The chance that a person or animal will encounter both members of a potentiating pair is smaller than the chance of encountering either one separately. This is especially true because, to be effective, the two compounds must be absorbed at about the same time and at dosages not very different from those that would be dangerous if only a single compound were absorbed. Traces are not effective.

Potentiation may be of critical importance in isolated instances, but it is virtually impossible to predict it, partly because there may be no apparent-and therefore predictive-pharmacological relationship between the two compounds involved and also because the mechanism of their interaction (Section 1.5.3.2) may not be known initially. An exception involves the organic phosphorus insecticides. DuBois *et al*. (1968) developed a quantitative procedure for measuring the potency of these compounds to inhibit aliesterases and amidases that are critical to their detoxification. DuBois (1972) has suggested that the use of this procedure constitutes a practical method of determining the dietary levels that might potentiate the toxicity of pharmacologically active compounds normally detoxified by esterases.

A factor greater than 100 was found for potentiation between malathion and triorthocresyl phosphate (TCP) (Murphy *et al*., 1959). Although TCP is an organic phosphorus compound, it is not a pesticide. Potentiating compounds need not belong to the same chemical class. An example is the potentiation of the toxicity of parathion by chlorophenothiazine (Gaines, 1962). Furthermore, the chance of encountering two compounds at about the same time is not always random. Striking exceptions are compounds used in related procedures, including drugs taken concurrently or used to treat intoxication. The reason for emphasizing drugs is that many are taken at dosages that equal or exceed the daily dosages of pesticides absorbed by the most exposed workers. The exact opposite of potentiation is expected when exposure to a toxicant and its antidote are associated either for prophylaxis or treatment. However, less thoroughly studied combinations of toxicants and drugs might prove to be potentiating, particularly if the drug is taken once the toxicant has been absorbed in sufficient dosage to produce illness. In this situation, even a moderate degree of potentiation might prove critical.

It is important that the clinician is aware of the possibility of interaction and that appropriate studies be made in all cases in which poisoning appears to have occurred but in which the degree of known exposure seems inadequate to account for the observed effect.

1.5.3.2 Mechanisms of Interaction

Compounds interact in the body by a wide range of mechanisms, including chelation, alteration of ionization, alteration of protein binding, and the inhibition, reactivation, or induction of enzymes. Original access to the body may be altered by some of these same mechanisms or by solvents, ion-exchange resins or absorptive colloids, or a change of the intestinal flora. The final pharmacological or toxicological effect of one or more interactions usually cannot be predicted except by careful study of a pair of compounds. The mechanisms involved in interaction of compounds are described at greater length in other sections.

The possible complexity of interactions must be emphasized. For example, calcium disodium EDTA is useful for removing lead from the body, but treatment that is too intense or prolonged or that employs certain other chelating agents can cause injury by disturbing the distribution of essential trace metals in the tissues. In their net effects, charcoal and ion-exchange resins are similar to chelating agents.

The discussion of protein binding includes an illustration of competition for binding sites as the basis for the interaction of two compounds.

The action of several pesticides depends on the inhibition of enzymes. The success of several antidotes depends on their ability to reactivate these inhibited enzymes. Thus ethylenediaminotetracetic acid (EDTA), British anti-Lewisite (BAL), and other chelating agents may restore enzymes blocked by heavy metals. Oximes such as pralidoxime chloride (2-PAM) may restore enzymes blocked by organic phosphorus compounds. Combined use of nitrites and sodium thiosulfate releases cytochrome oxidase blocked by cyanide.

Enzyme Induction When the same substrate and enzyme are involved, inhibition and induction have opposite pharmacological effects. Thus, inhibition of liver S-desulfurase by SKF 525-A or by feeding a protein-free diet antagonizes the action of azinphosmethyl. Conversely, induction of the same enzyme by 3-methylcholanthiene or 3, 4-benzpyrene potentiates the action of the insecticide (Murphy and DuBois, 1958). Under different circumstances (especially the involvement of an enzyme of opposite pharmacological action) the effect of inhibition and induction may be reversed. For example, inhibition of liver aliesterase by EPN, TCP, or a number of other compounds potentiates the action of malathion (Murphy *et al*., 1959; Murphy and Cheever, 1968).

Perhaps the decision tree will help to sort out some of the most difficult problems of toxicology and that is how to deal with mixtures of chemicals. Part of the problem might be that in spite of widespread awareness of kinetic interactions there is a nearly complete absence of kinetic considerations when interactions between chemicals are viewed, as exemplified by the preceding subsections or by other comprehensive reviews (Pöch, 1993). No attempt will be made here to address the issues of potentiation, synergism, and other complex interactions, which may be of phenomenological origin and therefore of limited theoretical interest. Keeping these conceptual restrictions in mind, there are two possibilities: two or more chemicals either do interact at an experimentally measurable level or else are considered to act independently. Independent action allows for a simplified safety assessment in that determining the safety of the most toxic component ($c_{threshold} = k/t_{lifespan}$) will automatically provide protection for all other constituents of the mixture, because there are no such dose responses that would make chemicals more potent at low than at high doses (Figure 1.5). When two or more chemicals do interact, they can act in concert (agonists) or against each other (antagonists). The interaction can have predominantely kinetic or mainly dynamic elements, with the possibility of interactions between the two, which probably gave rise to the notion of potentiation, synergism, etc.

Kinetics One chemical can potentially affect any step in the disposition of another chemical, leading to kinetic agonism or antagonism. For example, it has been demonstrated clinically that administration of a penicillin-sulfonamide mixture to premature infants resulted in kernicterus, because the sulfonamide displaced bilirubin from its albumin binding site, increasing the free fraction of bilirubin in plasma, making it thereby available for diffusion into the brain (Silverman *et al*., 1956). Kinetic antagonism leading to therapeutic agonism was demonstrated in the early days of penicillin when penicillin was administered together with probenecid to block its active tubular secretion. Of toxicological relevance is also the fact that some organic acids compete with uric acid for the same organic acid transporter in the kidney and thereby can precipitate an acute attack of gout. Still another example is the increased nonbiliary intestinal excretion of some lipophilic chemicals across the gastrointestinal wall by oral administration of mineral oil or the trapping of biliary metabolites by cationic or anionic resins mixed with the feed (Rozman, 1986). Dietary constituents, vehicles, etc. often significantly alter absorption by either enhancing (kinetic agonism) or reducing (kinetic antagonism) it. These examples represent a tiny fraction of what is known about kinetic interactions between chemicals in mixtures. Yet we (Rozman and Doull) are not aware of any attempt to conceptualize the role of kinetics in the toxicity of mixtures.

Dynamics The effect itself is always of primary interest, even when a kinetic process(es) represents the rate-determining (-limiting) step(s) (see Section 1.1.1 for the fundamental equation of toxicology). Nevertheless, the nearly complete absence of kinetic considerations when conducting tier testing (NRC, 1988) or constructing isobolograms (Pöch, 1993) is lamentable and is probably part of the reason neither one is working particularly well in any other than specific situations. Classical agonistic and antagonistic interactions of binary and some ternary mixtures of drugs and other chemicals have been described so many times that even a superficial discussion of this topic appears unnecessary. The critical question though remains and concerns the mechanism of action. Chemicals exerting their toxicity by independent mechanisms can be dealt with by identifying the most toxic component and establishing a safety assessment for this compound, which will provide safety for all other, less potent constituents of the mixture. Chemicals acting by the same mechanism will display additivity in their effect. Antagonistic effects occur seldom because mixtures seldom contain comparable relative concentrations of an agonist and an antagonist. In addition, if the kinetics of the agonist and antagonist are very different, any potential interaction may turn out to be insignificant.

Supraadditivity usually and perhaps always amounts to a lack of understanding of the interaction. For example, the well-known 3-10-fold synergism in organophosphate (OP) poisonings is almost certainly due to two variables, one being the ratio of the half-lives of the two OPs, the other being related to the nonspecific detoxification pool (plasma carboxyesterases and other high affinity proteins in tissues). If both OPs are administered as single compounds, a large percentage of the dose of each will be detoxified by plasma carboxyesterases and only a fraction of the dose will reach the primary targets of acute toxicity (central nervous system, lungs, diaphragm). However, if one OP is administered before the other, then (depending on timing) the second OP will encounter varying degrees of occupation of the detoxification sites in plasma and therefore a larger portion of its dose will be available to exert toxicity at the target sites. It is our conviction that, although it may not be possible to explain potentiation and synergism on grounds of dose responses alone, but we may very well be able to do so in terms of both dose and time as variables of the interaction.

1.5.3.3 Interactions that May Influence Laboratory Tests

Most commercial products that are used as pesticides are marketed as formulations and thus contain vehicles and other ingredients that give the pesticide the desired properties for its intended use. These materials that are added to the active ingredients to provide the proper physical characteristics

cannot be assumed to be inert; these materials have toxicological properties of their own that are, in some cases, of greater importance than the toxicological properties of the active ingredient itself. In some of those cases, the toxicity of the total formulation reflects that of the active ingredient only because it is there in larger quantity than the other ingredients. Similarly, in toxicological studies, vehicles and other materials that may be added to a test agent have some influence on its toxicity. Except for studies of toxicity to the eye, most toxicological studies do not use the application of chemicals in their pure form. Inhalation toxicology studies may be done on a pure chemical, but often are conducted using technical products or commercial products, which have stabilizers or other ingredients that may or may not influence the toxicity of the chemical under test. In toxicological studies by other routes of administration, vehicles of one kind or another are routinely used to provide good mixing in diets or drinking water or to provide consistency of dose volume and ease of measurement of doses administered. The actual vehicle used varies widely by type of study, route of administration, nature of the chemical to be administered, as well as geographic region of the world. In the United States, the most commonly used vehicles are water and corn oil. Because most drugs and other organic chemicals in commercial use are not very soluble in water, an oil-based vehicle is widely used commercially and in toxicology laboratories.

Effect of Formulation The toxicity of a compound may be modified by differences in formulation. Solvents are especially important in this connection, but wetting agents and other ancillary compounds may be involved. When these chemicals promote or retard the toxicity of a pesticide, it is usually through promotion or retardation of absorption. The facilitating action may involve injury to a barrier, especially in the skin. Increase in absorption may also involve a solvent that, by its own ready absorption, enhances absorption of the toxicant.

Importance of Environmental Chemicals The source of a compound that influences the toxicological or pharmacological action of a recognized compound is not always obvious. A striking example is the alteration of drug metabolism in rats and mice by cedarwood bedding in their cages (Ferguson, 1966; Vesell, 1967; Wade *et al*., 1968). Another example is the change in reaction to molybdenum caused by traces of zinc derived from galvanized cages (Section 1.5.11.4). An example of a toxic interaction from human experience that was at first obscure is that of asbestos dust and cigarette smoke.

The identity of some other interactants is obvious. The concentration of ammonia fumes in the air of animal rooms from bedding soiled with urine has occasionally been a source of complaint by personnel working in the animal rooms. It is also now recognized as a possible complicating factor in the interpretation of animal studies, particularly when there might be respiratory lesions. Broderson *et al*. (1976) evaluated the effects of ammonia at concentrations of 25–250 ppm in the air of animal rooms on the characteristics of murine respiratory mycoplasmosis in Sherman and Fischer rats. The prevalence of pneumonia, but not of other respiratory lesions of murine respiratory mycoplasmosis, showed a strong tendency to increase directly with environmental ammonia concentrations. Exposure to ammonia of rats that had not been infected with the mycoplasma organism caused anatomic lesions that were unlike those of mycoplasmosis and were limited to the nasal passages. The authors concluded that environmental ammonia at concentrations commonly encountered in cage environments for rats played an important role in the pathogenesis of murine respiratory mycoplasmosis.

Some information on detectable concentrations of bacterial toxins, heavy metals, solvents, pesticides, and other environmental contaminants in laboratory feed and drinking water is available (Newell, 1980; Rao and Knapka, 1987; Rao, 1986; Williams, 1984).

1.5.4 Schedule of Dosage

It is common knowledge among toxicologists that the schedule of dosage may have an important influence on the quantitative results. Usually anything that permits greater detoxification or excretion of a toxin tends to reduce the injury it produces. An oral dose given on an empty stomach is absorbed over a briefer period than the same dose administered when the stomach is at least partly full. Ingestion of a certain daily dosage mixed in the diet often is less injurious than the same dosage of the same compound administered daily by stomach tube. The compound reaches a lower maximal concentration in blood and other tissues when the same dosage is distributed throughout the day rather than concentrated in a brief period of time. The microsomal enzymes, excretion, and other defenses may be able to cope indefinitely with a low concentration of a compound but may not be capable of handling peak levels.

Similar reasoning applies to schedules that permit rest periods as compared with those that do not. Truly continuous exposure is usually more damaging than intermittent exposure at the same daily rate. An example may be cited for lead (Kehoe, 1961). It must be noted, however, that the distinction between continuous and intermittent exposure is blurred somewhat for a compound that is stored, such as lead.

An even more dramatic example involves carbon tetrachloride studied in connection with the possible continuous exposure of people in submarine vessels or stations. It was found that intermittent exposure (8 h/day, 5 days/week) to carbon tetrachloride at a concentration of 515 mg/m^3

killed a small proportion of experimental animals and caused injury, especially to the liver, of many of those that survived for 6 weeks. About the same degree of injury was produced by continuous exposure at a concentration of only 61 mg/m^3, and this occurred within about the first 6 weeks (Prendergast *et al*., 1967). Thus, under these conditions, continuous exposure was about eight times more dangerous based on concentration and twice as dangerous based on total dose than intermittent exposure similar in schedule to much occupational exposure.

The effects of different schedules of dosing may differ qualitatively as well as quantitatively, sometimes in such a way that intermittent exposure to a concentration too high to tolerate continuously leads to a greater variety of pathology than is seen under any other condition. Thus, Landry *et al*. (1985) reported that mice exposed to methyl chloride at 2400 ppm for only 5.5 h/day showed renal pathology, intravascular hemolysis, and hematopoietic effects in addition to the cerebellar granular cell degeneration and consequent neuromuscular dysfunction seen in mice exposed to lower concentration on the same schedule or in those exposed to any of a range of concentrations for 22 h/day.

The cited work by Prendergast and his colleagues and earlier related work by the same and other groups of investigators offer some indication that a considerably smaller dosage of each of a number of compounds is required to cause injury if exposure is continuous rather than intermittent. Unfortunately, many of the investigations are reported in such a way that a meaningful comparison is impossible. Because many people are exposed to air pollution, some of them continuously, it seems tragic not to compare the effects of continuous and intermittent exposure at equal intervals of time after initial exposure. This is particularly true because the equipment and procedure for continuous exposure are specialized and costly. As long as tests are to be done, little difficulty or expense would be added by gathering and presenting comparable data. Certainly the number of persons now exposed continuously to ordinary air pollution is vast compared with the number who will enter the closed atmospheres of spacecraft or submarine vessels or stations in the foreseeable future.

The general rule that rest periods and avoidance of peak blood levels tend to be protective usually applies most to compounds that are easily detoxified and excreted, and least to compounds against which the defenses of the body are inherently poor, with the result that the compounds or their effects are relatively cumulative. The cumulation may occur over relatively long periods as, for example, with lead, or over short periods as, for example, with carbon monoxide. In the former case, the cumulation frequently involves months. In the latter case, the cumulative effect may involve hours or days, but is not prominent in connection with longer periods. All of us inhale carbon monoxide, and those of us who smoke tobacco inhale more than nonsmokers (Hanson and Hastings, 1933). Some garage workers encounter a level of exposure that is marginal with respect to injury. Higher levels of exposure involve progressively more hazard with the result that in some countries carbon monoxide kills more people than any other single compound.

In some instances (Saffiotti and Shubik, 1956; Taylor and Nettesheim, 1975; Waud *et al*., 1958) repeated small doses produce greater effects than a smaller number of larger doses even though the total dosage resulting from the larger number of applications is the same or less. This relationship would appear to violate a dosage-response relationship. The explanation may involve a failure of one or a few doses to reach the target tissue. Prolonged action may be required when there is an inherent delay between the initial dose and first observed effect regardless of whether this effect follows one or more doses. The apparently inverted dosage response may involve a purely pharmacological effect such as the depletion of tissue norepinephrine by reserpine (Waud *et al*., 1958) or it may involve toxic effects as discussed in Section 1.3.3.2.

The modifying effect of schedule and a number of other factors must be taken into account in any considerations of dosage. Tests to establish safe levels should involve the dosage schedule, route, and other conditions people are expected to encounter. When these modifying factors are taken into account, the paramount importance of dosage becomes even more evident.

This section illustrates best how well outstanding toxicologists understood the factors influencing toxicity. Yet, it was not possible to take this discipline to the next level without recognizing time as a independent quantitative and quantifiable variable of toxicity.

The example of carbon tetrachloride toxicity in rats used by Hayes is a good case to illustrate the power of examining both dose and time as variables of toxicity. The half-life of carbon tetrachloride is about 7 h (ATSDR, 2005). Exposure to 515 mg/m^3 for 8 h means that rats were continuously exposed to this compound for 24 h/6 weeks above about 130 mg/m^3, which is clearly above the toxicity threshold because 61 mg/m^3 for 8 h/6 weeks also caused moderate liver pathology. However, the later exposure dropped to about 12 ppm after every day's 8 h exposure, which was just slightly above the 7 h hepatic toxicity threshold (=8 ppm) (see ATSDR, 2005) and below the 6 h/day for 4 days LOAEL of 50 ppm. Therefore, it is very obvious that at the higher dose rate (515 mg/m^3) rats were afforded no time for recovery between exposure episodes, whereas at the lower dose rate they had plenty of time for recovery. Haye's other examples are similarly easy to explain using the theory of toxicology.

1.5.5 Duration of Dosage

Weil and McCollister (1963) investigated the degree of toxicity revealed by short-term and long-term tests in rats.

For 22 compounds, the ratios of the dosages producing the minimal effect observed in short-term and in 2-year feeding tests varied from 0.5 to 20.0 and averaged 2.9. Ratios greater than 1.0 indicated the degree of apparent increase in toxicity associated with long-term testing. Ratios less then 1.0 may have indicated adaptation, experimental variation, or both. It was possible to compare the maximal dosages of 33 compounds producing no effect; these ratios ranged from 0.5 to 12.0 and averaged 2.3.

Some methods for measuring the effects of duration of dosage are discussed in Sections 1.3.1.2, 1.3.1.3, 1.3.2, and 1.3.3.2.

Similar studies were continued by Weil *et al*. (1969) and expressed in somewhat different terms. The comparisons involved 20 compounds, including 11 pesticides. The LD 50 values were determined and the compounds were fed to rats for 7 and 90 days, respectively. The results are compared with those in 2-year studies done earlier. The LD 50 values offered a poor indication of the results of repeated dosing. However, the results of long-term exposure could be predicted in an efficient way from the results of exposure lasting only 7 days. Using subscripts to indicate the number of days of exposure, it was found that the relationships for predicting the lowest dosage large enough to produce a minimal effect (MiE) were those shown in Table 1.6.

Littlefield and Gaylor (1985) showed that under conditions of the study, daily dosage rate (mg/kg/day) seemed to be more important than duration of dosing in increasing the prevalence of liver and bladder tumors in mice fed 2-AAF when the total dosage was the same (Rozman and Doull, 2001a).

1.5.6 Route of Exposure

The route by which a compound is absorbed helps to determine not only the ease of absorption, but also, in some instances, the ease of metabolism. Compounds are usually more toxic by the oral then by the dermal route. This was true of 64 of 67 compounds studied by Gaines (1960, 1969) and analyzed in this regard by Hayes (1967a). However, there were three exceptions, that is, three compounds more toxic by the dermal route. Considering all 67 compounds, the factor of difference by which oral toxicity exceeded dermal toxicity raged from 0.2 to 21 and averaged 4.2.

TABLE 1.6 Ratios for Predicting the Results of Long-Term Feeding from the Results of Short-Term Feeding[a]

Value	Ratios for predicting result of	
	90-day feeding study	2-year feeding study
Median value	$MiE_7/3.0$	$MiE_{90}/1.8$ or $MiE_7/5.4$
95th percentile	$MiE_7/6.2$	$MiE_{90}/5.7$ or $MiE_7/35.3$

[a]*Modified from Weil et al. (1969), by permission of Academic Press.*

The lesser toxicity of one of the compounds (isolan) by the oral route was markedly influenced by metabolism. Five of six rats survived infusion of isolan into an intestinal vein for an hour at a rate that led to death within 18–35 minutes in six comparable animals infused via the femoral vein (Gaines *et al*., 1966). Thus, a single pass through the liver is sufficient to make the difference between life and death as a result of exposure to isolan. This phenomenon helps to explain the high dermal and low oral toxicity of the compound. The high dermal toxicity of monochloracetic acid was due to irritation-related rapid absorption and the much lower oral toxicity occurred because of delayed stomach emptying, also due to local irritation (Saghir and Rozman, 2003).

Gaines (1969) found that about one-third of the pesticides he tested had such a low or variable dermal toxicity that no LD 50 could be determined. Thus, the true average difference between oral and dermal toxicity is greater than that calculated for compounds for which definite dermal as well as oral LD 50 values can be measured. Furthermore, relatively low dermal toxicity may be characteristic of an even higher proportion of compounds generally than is true of pesticides.

For practical reasons, respiratory toxicity usually is studied and reported in terms of concentration of chemical and duration of exposure. Values obtained in this way cannot be converted easily to dosage in terms of body weight, and direct comparison with toxicity by other routes is not usually possible. Some notion of the respiratory toxicity of a compound may be inferred from its intravenous toxicity (DuBois and Geiling, 1959). However, the method is of limited value partly because gases and aerosols are absorbed by the respiratory tract to different degrees depending on the compound and the particle size.

The route of administration may have a clear effect on the delayed neurotoxicity induced by certain organophosphorous esters. As summarized by Francis (1983), TCP caused delayed neurotoxicity in rhesus and squirrel monkeys and dogs when given by subcutaneous administration but not by the oral route. Mipafox caused a positive response when given to rats subcutaneously but caused only an equivocal response when given in the diet. These differences may have been related to differences in metabolism involving a single pass through the liver.

The differential in response from two different modes of oral administration, diet versus gavage, was evaluated by Weil *et al*. (1973). Rats in a reproduction study and guinea pigs in a teratology study were given carbaryl by one of the modes of oral exposure. Maximal dosage levels in the reproduction study were 200 mg/kg/day by diet or 100 mg/kg/day by gavage. Maximal dosage levels in the teratology study were 300 mg/kg by diet or 200 mg/kg by gavage. The maximal gavage groups had severe maternal toxicity in contrast to little or no effect in the diet groups

that received higher dosages. Thus, differences in the mode of oral administration (gavage versus diet), which is essentially a difference in schedule of dosage, can be as important as differences between routes of exposure.

Another clear example of an effect of route of administration and dosage schedule on toxicity is a study reported by Taylor and Nettesheim (1975) regarding the evaluation of the carcinogenicity of nitrosoheptamethyleneimine. F344 and Sprague-Dawley rats were given this chemical by gavage or subcutaneous injection; cumulative dosages ranged from 5.5 to 1200 mg/kg and the dosage schedule ranged from 40 serial administrations to one single injection. Oral administration was more effective in producing tumors than was subcutaneous injection at approximately the same total dosage, and administration of multiple small doses was more effective than a single large dose when the total dosage was constant.

All three preceding subsections relate to time as a variable of toxicity. As such, they belong together with Section 1.5.1 (dose) dealing with quantitative variables of toxicity. Schedule and duration of dosing has been combined and expressed as exposure frequency (Section 1.1.1) whereas the route of exposure is related to the timescale of absorption. These topics have been discussed in great detail in Section 1.1.1 and here it should suffice to illustrate this with still another example how the theory of toxicology together with the decision tree helps explain experiments that were misinterpreted by both toxicologists and nontoxicologists. Littlefield and Gaylor (1985)'s interpretation of another part of the ED 01 study is a good example of nontoxicologists addressing issues they do not understand. This particular paper claims that the daily dose (dose rate) is more important in the bladder carcinogenicity of 2-AAF than is the total dose (dose), because mice receiving a high dose rate (150 ppm) of the compound for 9 months had a higher cancer rate at 18 and 24 months than those given a lower dose rate (60 ppm) for 24 months.

First, the dose (total dose) administered to the mice was comparable in the two groups of animals as demonstrated by the AUCs of exposure of 150 ppm $\times$ 9 months = 1350 ppm $\bullet$ months for a high dose group or 60 ppm $\times$ 24 months = 1440 ppm $\bullet$ months for a low dose group of animals. Yet mice in the lower dose rate group had 1% bladder cancer each at 18 and 24 months, whereas mice in the higher dose rate group after 6 and 12 months recovery still had much higher cancer rates (6 and 18%, respectively). This finding is entirely consistent with the theory of toxicology. Polycycic aromatic hydrocarbons have short kinetic half-lives (about 1 day or less), but long dynamic (recovery) half-lives which dominate (rate-determining step) their action. Thus, the dynamic AUC kept growing for the group for which dosing was stopped at 9 months and as a consequence bladder cancer incidence increased from 6% to 18% during the period lasting for 18–24 months or for any of the time periods after cessation of dosing (Littlefield and Gaylor, 1985). This increase was entirely consistent without regard to whether dosing was stopped at 9, 12, 15, or 18 months into dosing. Thus, it is very clear that the higher dose rate generated a much larger dynamic AUC than the lower dose rate. Deducting the threshold AUC from both the high and low dose dynamic AUCs indicates that 150 ppm 2-AAF administered for 9 months is 5–10 times more effective after 18 and 24 months in terms of dynamic AUC than is 60 ppm 2-AAF administered continuously for these periods of time. This example is a further illustration of how the theory of toxicology allows for a biologically plausible interpretation of results that, in the past, usually were submerged in a maze of biologically implausible but formalistically correct statistical gibberish.

1.5.7 Species and Strain Differences

This section deals almost exclusively with dynamic aspects of species and strain differences with only peripheral reference to species differences that are due to differential kinetics, although knowledge of the latter is very widespread. This discrepancy illustrates that, unless knowledge is conceptualized in the framework of a theory, its application remains haphazard.

1.5.7.1 Species Differences Due to Dynamics

It is well recognized that species differences impose considerable limitations on our ability to predict the toxicity of a compound from one species to another. The dismal state that has resulted from predicting that a compound found to be carcinogenic in the rat will also be carcinogenic in the mouse and vice versa is a case in point (DiCarlo, 1984; DiCarlo and Fung, 1984). Therefore, many scientists and even more nonscientists question the value of whole-animal studies for the protection of the public from potential adverse health effects of drugs and other chemicals.

Most of the scientific arguments in support of moving away from whole-animal studies are firmly rooted in the prevailing reductionist thinking of the 20th century. The quintessence of these arguments is an irrational hope that molecular events at the level of DNA and RNA and/or at high affinity protein binding sites will eventually explain everything that goes awry in an organism as a result of a toxic insult.

In sharp contrast and with barely audible voice at present are a few antireductionists, who despair at the complexity of a mammalian organism. In their view, the multitude of causes for species differences in toxicology precludes the possibility of resolving these issues. However, whole animals still represent a more valid biological system for comparison and prediction because of some qualitative similarities between species.

Both sides, of course, have some valid points, but they miss many more. The overwhelming importance of genetics in the inherent capability of a cell to respond to a toxic insult has been demonstrated amply. However, the cell is not a pile of molecules and an organism is not a random cell culture, rather, cells are organized in tissues in a *hierarchial* fashion with implicit rank order of importance for the organism. Moreover, there is a flow of information not only between adjacent cells but also between tissues. Therefore, the question of how a particular species will respond to a toxic insult depends not only on the interaction between a xenobiotic and a subcellular element, but also on the hierarchical status of the target tissue and on the possible disturbance of information flow between tissues. Having said this much, it is clear that a resolution of species differences will have to involve both analytical and synthetic thinking. Moreover, although understanding of species differences is important, it is so only to the extent of defining similarity (species- or strain-reactivity) between species to allow meaningful interpretation of results from one species to another.

Differences between Parasites and Hosts There is a tendency to ignore, as objects of scientific interest and wonder, the differences in the susceptibility of pests and of organisms we hope to protect. This attitude may be justified if the difference is based more on difference in exposure than on inherent susceptibility. The difference cannot be ignored when it involves "systemics," that is, pesticides used as drugs to combat parasites on or in their host. Examples of systemics for mammals include crufomate, trichlorfon (metrifonate), dichlorvos, ronnel, diphacinone (diphenadione), and coumaphos. Some of these compounds were originally developed to destroy botflies that pass their larval stages in the tissues of cattle, the mucous membranes or nasal sinuses of sheep, or the stomach and anterior small intestines of horses. Ronnel is used as a systemic treatment for fleas and the action of dichlorvos on fleas may be partially systemic. During the early studies, it was found that control extended to some but not all species of nematodes, including some in the tissues rather than the intestinal lumen. Trichiorfon has been used to treat helminthiasis, including ankylosomiasis, ascariasis, trichuriasis, and creeping eruption in humans (Cerf *et al*., 1962). The expected pharmacological effects of the drug did appear as side effects but these effects were no more severe or frequent than those of other anthelminthics. Trichlorfon is effective for treating even *Schistosoma haematobium* infestation (King *et al*., 1988).

The control of botfly larvae and fleas is certainly due to the anticholinesterase and other antiesterase action of the drugs and no other mode of action is known against the susceptible nematodes. The fact that the compounds can be effective against parasites in the tissues without injuring the host is striking evidence of very great differences in susceptibility to absorbed drug. The mechanism of the difference is poorly understood but probably depends on the greater susceptibility of the esterases of the parasite and the greater metabolic power of the microsomal enzymes of the host.

(It may be noted that systemics for plants are compounds capable of being absorbed by one part of the plant and then translocated to another part so that the plant becomes pesticidal. Absorption usually occurs through the roots but may occur through the leaves or other plant organs.)

Differences between Vertebrates As a general rule, small species of warm-blooded animals eat more food than large ones in relation to their body weight. Therefore, if both kinds receive the same contaminated diet, the small species will receive a large dosage of toxicant.

However, the most notable examples of species differences do not depend on differences in food intake or respiratory pattern but are inherent. Sometimes the inherent difference may be explained in terms of metabolic or genetic differences. Very large differences in susceptibility are more likely among species belonging to different phyla but may occur among species of the same class. Norbormide is associated with a wide range of susceptibility among mammalian species. Albino Norway rats have an LD 50 of 4.3 mg/kg, whereas dogs, cats, monkeys, sheep, chickens, and turkeys are unaffected by single doses at the rate of 100 mg/kg (Roszkowski *et al*., 1964; Roszkowski, 1965). Thus, the factor of difference for these species for norbormide is greater than 23. Even the wild strain of Norway rat is less susceptible (LD 50, 12 mg/kg). Another example of marked species susceptibility involves ducks and diazinon.

Apparently the largest difference in species susceptibility that has been measured is that for the acute oral toxicity of TCDD in the guinea pig and hamster; this difference may be more than 8000-fold. This large species difference was so frightening that it gave rise to the most conservative risk assessment ever conducted (0.008 pg/kg/day daily intake), because the reason for this extremely large species difference was not understood. In the mean time it became clear that there is no such huge difference in terms of chronic toxicity and that the extraordinary difference in acute toxicity is due to differences in the way an herbivore (guinea pig) and a hibernator (hamster) can handle a disturbance of glucose metabolism. (Rozman, 1992; Fan and Rozman, 1994) The devastating lesson from the dioxin issue for toxicology is that having understood almost anything and everything about this class of compound at the cost of billions of the year 2,000 dollars has not changed the original risk assessment (based on very limited data) one iota. A cynical observer might conclude that there is an inverse relationship between toxicological investigation and "related" risk assessment.

Another very large difference involves the teratogenic effects of thalidomide. Human embryos have been deformed by as little as 0.5–1.0 mg/kg taken daily by the mother for

several days. At the other extreme, no injury to cat embryos was produced by a maternal dosage of 500 mg/kg/day, indicating a difference of more than 1000-fold. In fact, all other species studied require a dosage greater than that which is teratogenic in some women. The rabbit responds rather uniformly to dosages of 30–50 mg/kg/day, whereas many strains of rats fail to respond to 4000 mg/kg/day even though a dosage of 50 mg/kg/day is teratogenic in a few strains (Kalter, 1965). Schumacher *et al*. (1968b) reported that thalidomide given orally to rats was poorly absorbed. When the drug was given intravenously at a rate of 10 mg/kg/day, malformations and resorptions were observed (Schumacher *et al*., 1968a). However, the basic difference remains. According to Schardein (1985) all chemicals known to be teratogenic in humans except some coumarin derivatives have been shown to be teratogenic in laboratory animals. That does not guarantee that a new compound always will be tested in a susceptible species and by an effective route.

Perhaps the most diagrammatic difference in the susceptibility of species to a poison involves the use of diphacinone (called diphenadione as a drug) to control vampire bats that feed on cattle. When injected intraruminally at a rate of 10 mg/kg, as recommended, the compound is harmless to cattle but fatal to any bat that feeds within 72 hours of treatment (Mitchell, 1968). Whereas the exact degree of difference between these two species does not seem to have been measured, it clearly is substantial because the method has been in routine use for years without injury to cattle and with excellent control of vampire bats.

Appleman and Feron (1986) evaluated toxicity data from 66 compounds that were tested in only two species (rats and dogs) to determine how frequently the dog provided data that were substantially different in a qualitative or quantitative nature from those obtained in rats. In this evaluation, the rat was highly predictive of responses in the dog. Applications of a 10-fold margin to the rat data accounted for nearly all the differences between rats and dogs.

Some differences between species may be of such degree that they are essentially qualitative. Examples include the propensity of ducks to develop cataracts of the lens in response to dinitro compounds, or that of hens and humans to develop delayed but permanent paralysis in response to tri-o-cresylphosphate and some other organic phosphorus compounds. Although these are unusual situations, they emphasize how different one species can be from another.

Table 1.7 shows that, for many pesticides, the factor of difference in susceptibility of the mouse, guinea pig, rabbit, and dog ranges from 0.2 to 11.8 and averages close to 1.0 in comparison with the susceptibility of the male rat. The factor is close to 1.0 for many anticancer agents also (Freireich *et al*., 1966). The fact that the species difference is usually small is confirmed by comparisons based on the kind of test that may be applied to both people and experimental animals or on information obtained in connection with accidental exposure of humans. Comparisons of the susceptibility of humans and animals are shown in Tables 1.8 and 1.9. The tables indicate, for example, that humans are more susceptible than rats to lindane, about as susceptible as female rats to parathion, and distinctly less susceptible to warfarin than rats of either sex.

Although in many instances in which a direct comparison is possible there is no marked difference, the difference appears to be about 100-fold for a few compounds and over 1000-fold for thalidomide. Furthermore, the tables by necessity present phenomena that can be studied in laboratory animals. Much of the reason for conducting tests in humans is the existence of hypersensitivity, subjective responses, and other phenomena that do not lend themselves to study in animals.

TABLE 1.7 Relative Susceptibility of Different Species to Pesticides Based on Oral LD 50 Values[a] Using the Male Rat as a Standard[b]

Compound	Susceptibility factor[c]			
	Mouse	Guinea pig	Rabbit	Dog
Chlorobenzilate	1.4	—	—	—
DDT	0.3–0.8	0.3	0.3–0.5	—
Methoxychlor[d]	2.7–3.8	—	—	—
Lindane	1.0	0.7–0.9	0.4–1.5	—
Aldrin	0.9–1.2	1.2–1.6	0.5–1.1	0.4–0.8
Chlordane	0.8	—	1.1–3.4	—
Dieldrin	1.2	0.9–1.0	0.9–1.0	0.7–0.8
Endrin	—	0.5–2.7[e]	1.8–6.2	—
Heptachlor	0.6–1.5	0.8–0.9	—	—
Azinphosmethyl	1.6	0.2	—	—
Chlorthion®	0.7	—	—	—
Diazinon	0.9–1.4	0.3	0.8	—
Dimethoate	3.0–5.4	0.5–0.9	—	—
Dioxathion	—	—	—	1.1–11.8
Malathion	1.6–1.9	—	—	—
Methyl parathion	0.4	—	—	—
Mevinphos	0.9–1.6	—	—	—
Oxydemetonmethyl	1.0–2.5	0.3–0.6	—	—
Parathion	0.2–5.0	1.6–3.2	0.5–3.0	—
Phosphamidon	1.3	—	—	—

[a]Data from Gaines (1960), Lehman (1965).
[b]From Hayes (1967a), by permission of the Royal Society, London.
[c]A factor of less 1.0 indicates less susceptibility than that of the male rat; a factor greater than 1.0 indicates greater susceptibility.
[d]Both sexes.
[e]Approximate.

TABLE 1.8 Comparison of the Susceptibility of Humans and Other Animals to Certain Pesticides[a]

Compound	Species (sex)	Dosage (mg/kg)								References
		Largest without clinical effect	Smallest with clinical effect	Median CD 50	Smallest with serious effect	Largest nonfatal	Smallest fatal	LD 50	Uniformly fatal	
DDT	Human	—	6	10	16[b]	285[c]	—	—	—	Garrett (1947); Hsieh (1954); Neal *et al.* (1946); Velbinger (1947); Hayes (1959)
	Rat (F)	—	—	—	75	150	100	118	200	Gaines[d]
	Rat (M)	25	—	—	50	175	50	113	200	Gaines[d]
Lindane	Human	—	0.4[e]	0.4[e]	0.5[b]	—	—	—	—	Graeve and Herrnring (1951)
	Rat (F)	—	—	—	—	125	75	91	—	Gaines[d]
	Rat (M)	—	—	—	50	125	75	87	200	Gaines[d]
	Calf	2.5	—	—	5	—	—	—	—	Radeleff *et al.* (1955)
Chlordane	Human	—	—	—	32b,c	—	—	—	—	Dadey and Kammer (1953)
	Human	—	—	—	—	—	29–57	—	—	Hayes (1963)
	Infant	—	—	—	10[b]	—	—	—	—	Lensky and Evans (1952)
	Rat (F)	100	200	—	300	550	350	430	600	Gaines[d]
	Rat (M)	—	—	—	250	400	250	335	450	Gaines[d]
Dieldrin	Human	—	—	—	10	—	—	—	—	Princi (1952)
	Rat (M, F)	—	—	—	30	60	30	46	—	Gaines[d]
Endrin	Human	—	—	—	0.2	—	—	—	—	Davies and Lewis (1956)
	Rat (F)	—	—	—	6	10	6	7.5	—	Gaines[d]
	Rat (M)	—	—	—	10	25	10	17.8	30	Gaines[d]
Dichlorvos	Human	—	—	—	51	—	—	—	—	Hayes (1963)
	Rat (F)	—	—	—	—	100	37	56	125	Gaines[d]
	Rat (M)	—	—	—	—	125	75	80	150	Gaines[d]
	Cow	—	—	—	27	—	—	—	—	Tracy *et al.* (1960)
	Horse	—	—	—	25	—	—	—	—	Jackson *et al.* (1960)
Diazinon	Human	—	—	—	2.2[f]	—	—	—	—	Hayes (1963)
	Human	—	—	—	—	250	—	—	—	Bockel (1957)
	Rat (M)	—	—	—	200	300	200	250	350	Gaines[d]
	Calf	—	—	—	1	—	10	—	—	Radeleff *et al.* (1955)
Malathion	Human	—	—	—	—	200	71	—	—	Walters (1957); Paul (1960)
	Rat (F)	—	—	—	750	1250	750	1000	1500	Gaines[d]
	Rat (M)	500	—	—	1000	1750	1000	1375	2000	Gaines[d]
	Sheep	150	100	—	100	300	150	—	—	Radeleff *et al.* (1955)
Parathion	Human	—	—	—	—	6.4	2.0	—	13	Goldblatt (1950); Hayes (1963)
	Child	—	—	—	—	—	0.1	—	—	Kanagaratnam *et al.* (1960)
	Rat (F)	—	—	—	1	4.5	3.0	3.6	5	Gaines[d]
	Rat (M)	5.0	—	—	10	20	10	13.0	30	Gaines[d]
	Calf	—	—	—	0.5	—	1.5	—	—	Radeleff *et al.* (1955)
	Sheep	50	—	—	—	75	20	—	—	Radeleff *et al.* (1955)
	Steer	25	—	—	—	—	—	—	—	Radeleff *et al.* (1955)
TEPP	Human	—	0.05	—	3.5	—	—	—	—	Grob and Harvey (1949) Grob *et al.* (1950)
	Rat (M)	—	—	—	—	—	1.0	1.05	—	Gaines[d] Gaines[d]

[a] *From Hayes (1967a), by permission of the Royal Society, London. All doses are single and oral unless otherwise noted.*
[b] *Convulsions.*
[c] *Part of does vomited.*
[d] *Based partly on published papers (Gaines, 1960, 1969) and partly on the original data from which the papers were drawn.*
[e] *Three times a day for 3 days; highly dispersed formulations.*
[f] *Dermal.*

TABLE 1.9 Comparison of the Susceptibility of Humans and Other Animals to Repeated Doses of Certain Pesticides[a]

Compound	Species (sex)	Dosage (mg/kg/day)	Duration (days)	Results[b]	Reference
DDT	Human	0.5	>600	Increased storage; no clinical effect	Hayes *et al.* (1956)
	Rat (M, F)	0.24	161	Histopathological changes of the liver	Laug *et al.* (1950)
Methoxychlor	Human	2	56	No effect	Stein *et al.* (1965)
	Rat (M, F)	4.87	750	No effect level	Lehman (1965)
Demeton	Human	0.05	24	15% reduction of plasma ChE only	Moeller and Rider (1962b)
	Rat (F)	0.05	112	No significant depressions of ChE	Barnes and Denz (1954)
	Rat (F)	0.14	112	30% inhibition of ChE	Barnes and Denz (1954)
	Rat (F)	0.24	66	60% reduction of plasma; 40% reduction of RBC ChE	T. B. Gaines, unpublished results (1962)
	Dog (M, F)	0.025	168	No significant depression of ChE	Frawley and Fuyat (1957)
	Dog (M, F)	0.047	168	Significant depression of ChE	Frawley and Fuyat (1957)
Dimefox	Human	0.002	70	No effect on ChE	Edson (1964)
	Human	0.0034	70	25% reduction of whole blood ChE	Edson (1964)
	Rat (F)	0.024	28	About 50% inhibition of RBC ChE; no effect on plasma ChE	Edson (1964)
	Rat (F)	0.095	28	75% reduction of RBC ChE; 25% reduction of plasma ChE	Edson (1964)
	Rat (F)	0.475	28	Almost complete inhibition of RBC ChE; 75% reduction of plasma ChE	Edson (1964)
Dioxathion	Human	0.05–0.075	59	No inhibition of RBC and plasma ChE	Frawley *et al.* (1963)
	Human	0.15	28	Slight inhibition of plasma ChE; no effect on RBC ChE	Frawley *et al.* (1963)
	Rat (M, F)	0.22	91	No significant effect on ChE	Frawley *et al.* (1963)
	Rat (M, F)	0.78	91	Significant reduction of RBC and plasma ChE	Frawley *et al.* (1963)
	Dog (M, F)	0.25	12	Marked effect on plasma ChE; no effect on RBC ChE	Frawley *et al.* (1963)
	Dog (M, F)	0.8	12	Marked effect on plasma ChE; no effect on RBC ChE	Frawley *et al.* (1963)
Malathion	Human	0.34	56	Maximal reduction of 25% plasma and RBC ChE	Moeller and Rider (1962a)
	Rat (F)	3.2	90	29% reduction in RBC and no reduction of plasma ChE on 30th day; recovery by 90th day	T. B. Gaines, unpublished results (1968)
	Rat (M)	4.5	730	10–30% inhibition of plasma and RBC ChE	Hazleton and Holland (1953)
Methyl parathion	Human	0.1	24	15% reduction of plasma ChE only	Moeller and Rider (1962b)
	Dog (M, F)	0.94	84	Significant depression of plasma and RBC ChE	Williams *et al.* (1959)
Paration	Human	0.1	42	33% reduction of whole blood ChE; 16% inhibition of RBC ChE; 37% inhibition of plasma ChE	Edson (1964)
	Rat (F)	0.07	90	No effect	T. B. Gaines, unpublished results (1968)
	Rat (F)	0.26	84	80% reduction of RBC ChE; slight inhibition of plasma ChE	Edson (1964)
	Rat (F)	0.35	90	37% reduction of plasma and 44% reduction of RBC ChE	T. B. Gaines, unpublished results (1968)
	Dog	0.047	168	60% inhibition of plasma ChE	Frawley and Fuyat (1957)
	Pig	4.0	49	80% inhibition of RBC ChE; no inhibition of plasma ChE	Edson (1964)
Schradan	Human	0.014	44	25% reduction of blood ChE	Edson (1964)
	Human	0.06	60	77% inhibition of RBCD ChE; 50% inhibition of plasma ChE	Edson (1964)
	Rat (M, F)	0.045	112	Substantial reduction of ChE; no effect on plasma ChE	Edson (1964)

TABLE 1.9 (Continued)

Compound	Species (sex)	Dosage (mg/kg/day)	Duration (days)	Results[b]	Reference
	Rat (M)	0.22	14–85	Complete inhibition of RBC ChE	Edson (1964)
	pig (F)	0.1	102	55% inhibition of RBC ChE; slight reduction of plasma ChE	Edson (1964)
Arsenic trioxide	Human	0.44	?	Frequent mild poisoning	Sollmann (1957)
	Sheep	10	—	Tolerated without symptons	Reeves (1925)
	Horse	4.7	—	Tolerated without symptons	Reeves (1925)
Warfarin	Human	0.14	Indefinite	Maintenance therapeutic dose	Friedman (1959)
	Human	0.29–1.45	15	Hemorrhage in 12 people (4–70 yr) followed by recovery	Lange and Terveer (1954)
	Human	1.7	6	Hemorrhage in 22 yr man followed by recovery	Holmes and Love (1952)
	Human	0.83–2.06	15	Fatal to boy (19 yr) and girl (3 yr)	Lange and Terveer (1954)
	Rat (M, F)	0.08	40	Killed 5 of 10	Hayes and Gaines (1959)
	Rat (M, F)	0.39	15	Killed 10 of 10 rats	Hayes and Gaines (1959)
2,4-D	Human	14–37	18	Tolerated	Seabury (1963)
	Human	66	1	Coma, hyporeflexia, incontinence	Seabury (1963)
	Rat (F)	15	112	Tolerated	Hill and Carlisle (1947)
	Dog	9	84	Tolerated	Drill and Hiratzka (1953)

[a]From Hayes (1967a), by permission of the Royal Society, London. All doses are oral unless otherwise noted.
[b]RBC, red blood cells; ChE, cholinesterase.

Species differences may involve any parameter. McCully *et al*. (1968) found as much as 40-fold difference in tissue levels of DDT following a single oral dose at the rate of 10 mg/kg to rats, sheep, chickens, rabbits, and guinea pigs. The greatest difference for three routes (oral, intraperitoneal, and intramuscular) in any one species was five-fold.

Species differences in the effect of inhaled chemicals on pulmonary function complicate the use of concentration in air as an indicator of dosage. Studies with methyl bromide (Medinsky *et al*., 1985) and formaldehyde (Chang *et al*., 1981) revealed significant species differences in the response of pulmonary function to these two inhaled chemicals. Changes in pulmonary function were such that dosage was not simply a function of concentration, time of exposure, and normal minute volume of the species. Thus, without knowledge of the effect on pulmonary function, extrapolation between species is particularly difficult.

Certain animals lend themselves to testing for a limited number of specific forms of toxicity. Thus, hens are used to screen for possible neurotoxicity of organic phosphorus compounds, not only because they are highly susceptible to this injury, but because this susceptibility seems to resemble that of humans. In a similar way, ducks are used to test the tendency of dinitro compounds to cause cataracts.

The ideal scheme would include a species of experimental animal resembling humans so closely in susceptibility to poisons that any differences would be unimportant. Unfortunately, no such animal has been identified or is likely to exist. Monkeys and apes have been suggested. They are valuable for special purposes, but there is no convincing evidence that their average value is greater than that of any other laboratory animal. In some instances they are distinctly inferior to other animals. For example, when fed DDT, rhesus monkeys metabolize little or none to DDE, although both rats and humans form this compound readily (Durham *et al*., 1963).

Variation between species must be considered every time a new compound that has been properly tested in animals is used for the first time. It is frequently suggested that the tests be made in a large number of species. If the results in the different animals are similar, it is likely that human response to the compound will not be greatly different. If, however, there is a wide variation in the response of different species, then conservatism forces us to suppose,

until there is direct evidence to the contrary, that humans may be at least as sensitive as the most susceptible species.

No matter what the pattern of response in experimental animals is, the ultimate tests must be in humans. It is best that such tests be carried out under circumstances permitting scientific observations.

Strain Differences Strain differences in background disease and susceptibility to toxicants has influenced the selection of strains of laboratory animals for use in routine tests. For example, regarding models for carcinogenicity testing, strains of animals, even though in common use, have differences, which are problematic. Life spans differ significantly from one strain to another and must be taken into account in selecting the duration of chronic studies. Sprague-Dawley rats are characterized by a fairly high incidence of mammary tumors, and the life span of the male rat is commonly limited by the background incidence of renal disease. The F344 rat, though widely used in carcinogenesis studies, has an extremely high occurrence of interstitial cell tumors of the testis, precluding the use of the testis as an organ of evaluation of testicular tumors of that cell type. The $B6C3F_1$ mouse, particularly the male, has a high and variable incidence of liver tumors (Haseman *et al*., 1984). There is no single strain or species of laboratory animal that is clearly most predictive of chronic toxicity or carcinogenicity for humans. That statement can also be made for most other end points of toxicity. Thus, acceptance of one or multiple species of animal for testing must recognize the limitations of each species and strain, and interpretation of results must be made accordingly.

Individual Differences Individual differences are apparent in every toxicological test, including those carried out in people. A paper by Gaines (1969), in which he reports the acute oral toxicity of pesticides, shows that for 69 compounds the LD 50 value for male rats was 1.20–7.14-fold greater than the corresponding LD 01 value. The average factor of difference was 2.42. The corresponding factors of difference for the dermal toxicity of 42 pesticides were 1.37–14.93 with an average of 3.00. In other words, judged in this way, individual variation, although very real, is usually relatively small. In studies of storage and excretion, the greatest individual average excretion of malathion-derived material differed from the smallest individual average excretion at the same dosage by factors of only 2.2–8.7 for different groups of people (Hayes *et al*., 1960). Thus, the degree of difference was relatively constant in tests carried out at different dosage levels or at different times. A similar observation was made regarding the storage of DDT and the excretion of DDA in humans. In separate tests, the maximal storage of DDT was 1.3–5.9 times the minimal storage at the same dosage level. For a single dosage group, the maximal rate of excretion of DDA by one man in any one day was 18.0 times greater than his own minimal rate, and the difference between the lowest minimum and highest maximum within the group was a factor of 21.5 (Hayes *et al*., 1971). In all of these tests, the relative constancy of one individual compared with another was noted.

Although individual differences may be described in statistical terms, physiological understanding of these differences is lacking almost entirely. If a population is sufficiently heterozygous, the differences between individuals may depend on their genetic diversity. However, individual differences persist to some degree in a homozygous population (see Section 1.3.1.4 for discussion on Gaussian distribution). This is illustrated by the failure of the LD 50 values of four pesticides in a particular population of mice to change in the course of l2 or more generations even though each succeeding generation was bred from mice that had survived an LD 50 dose (Guthrie *et al*., 1971).

Sex Chiefly, because of its convenience, the rat is used more than any other species for studies in toxicology. The rat also has the apparent distinction of showing more variation between the sexes in its response to chemicals than any other species. This fact may have led to more concern than is justified regarding possible differences in the susceptibility of men and women to chemicals. In any event, calculations from data provided by Gaines (1960, 1969) for the oral toxicity of 69 pesticides showed that the difference in the oral LD 50 for male and female rats ranged from 0.21 (indicating greater susceptibility of the female) to 4.62 (indicating greater susceptibility of the male), and averaged 0.94. The corresponding factors for the dermal toxicity of 37 pesticides were from 0.11 to 2.93, with an average of 0.81 (Hayes, 1967a). The differences in the susceptibility of male and female rats are associated to a large degree with differences in their liver microsomal enzymes.

In contrast to the situation in rats and to a lesser degree in other rodents, significant differences between the sexes of other species in their susceptibility to poisons usually have not been reported. Such differences were looked for but not found in studies of the storage of DDT in monkeys (Durham *et al*., 1963; Ortega *et al*., 1956). Such differences between men and women are small or lacking entirely.

Pregnancy Susceptibility to a particular compound may be either greater, less, or identical in pregnant females than in nonpregnant ones of the same strain and age. For example, pregnancy exaggerates the danger of anticoagulants but reduces the danger of paraquat. For some differences such as susceptibility to anticoagulants the reason for the difference is clear. In most instances the reason is obscure.

In a systematic study of 19 drugs, given by different routes, pregnant mice were more susceptible than nonpregnant ones by factors ranging from 0.74 to 14.55 and averaging 1.90, or 1.27 if the single high value is excluded (Beliles, 1972).

Lactating rats consume approximately 3-fold (Hayes, 1976) or 2.5–3-fold (Yang *et al.,* l984a) more feed than the same rats before or after lactation; thus lactating rats

are subject to a marked increase in the dosage of all compounds in their diet. The extent to which this is true of other species apparently is not documented.

Other Endocrines There is considerable evidence that the pituitary adrenal axis may be influenced by photoperiodicity and in turn may influence susceptibility to toxicants.

Lipsett (1983) has reviewed the relationship between hormones and cancer. Sex and other hormones usually have either pulsatile release or some cyclicity (diurnal or circadian cycles) in their secretion. In either event, an additional timescale needs to be incorporated into studying these phenomena in the context of toxicity in addition to the three independent timescales discussed earlier. Gender differences will become manifest in toxicology only when the aforementioned timescale becomes rate-determining (or-limiting). Pregnancy introduces still another timescale with known consequences for altered hormonal timescales for a limited period in an individual's life.

Age Children and young animals are often more susceptible than adults to poisons in food. The most common reason is that children and other young animals eat more than adults in proportion to their weight. Thus, when given the same contaminated food, young animals receive a higher dosage of toxicant. The relationship for rats is shown in Fig. 1.19. Although the figure is based on DDT, it applies equally to any compound that does not cause a reduction of food intake.

However, other factors may be involved. It is now well known that a number of drugs are poorly metabolized by infants, particularly those born prematurely (Fouts and Adamson, 1959). Although it is seldom possible to quantitate the difference, it is clear that a dosage of some drugs easily tolerated by human adults may lead to severe illness or even death in very young children.

Calves and sometimes lambs are markedly more susceptible than adult cattle or sheep to sprays or dips of chlordane, dieldrin, and lindane (Radeleff, 1970).

Systemic study of drugs (Hoppe *et al*., 1965; Yeary and Benish, 1965) and pesticides (Brodeur and DuBois, 1963; Gaines and Linder, 1986; Lu *et al*., 1965) indicates that newborn animals are generally more susceptible than adults of the same species regardless of route of administration. However, the differences tend to be small and there are some exceptions in which the newborns are actually less susceptible than adults (Brodeur and DuBois, 1963; Gaines and Linder, 1986; Hoppe *et al*., 1965). As reviewed by Durham (1969), the difference, no matter what its direction, can often be explained in terms of the recognized activity of microsomal enzymes in activating or deactivating the chemical in question. Other factors that may be of importance are renal function and membrane permeability. Fortunately, compounds of similar pharmacological action tend to show similar differences in their toxicity to young adult animals (Yeary and Benish, 1965). In studying drugs, Yeary and Benish found that newborn rats were 0.6–10.0 times more susceptible than adults. Hoppe *et al*. (1965) found a similar range of 0.7–6.2. Goldenthal

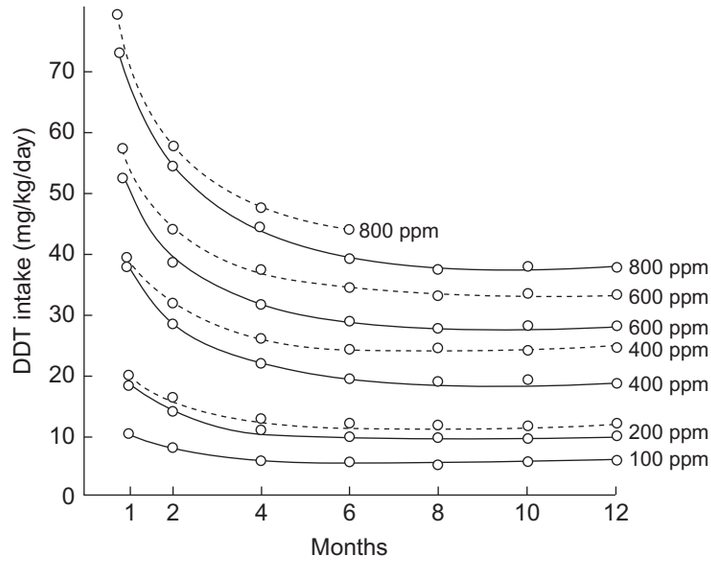

FIGURE 1.19 Calculated DDT intake (mg/kg body weight) in rats receiving various levels of DDT in the die (male, —; female,---). From Fitzhugh and Nelson (1947), by permission of the Williams & Wilkins Co., Baltimore.

(1971) studied a much larger number of drugs and a few other compounds (290 in all) and reported a much wider range of factors of differences: <0.2–750. However, by omitting only one low factor and eight high ones, the range was narrowed to 0.1–20. The geometric mean of all of the factors (no exceptions) was 2.78.

In their studies of 15 organic phosphorus insecticides or defoliants, Brodeur and DuBois (1963) found that weanling male rats were 0.2–4.1 times more susceptible than adults, with a mean variation of 1.8 DDT is also less toxic to infant rats than to adults.

Age-related differences in susceptibility to carcinogens have not been given extensive attention, but there is increasing concern about the potential for transplacental carcinogenesis (carcinogenic effects associated with *in utero* exposure). Few chemicals are known to be carcinogenic by *in utero* exposure only (and not from lifetime or long-term exposure after weaning); however, in certain cases the profile of tumors is known to be different between the two exposure patterns, for instance, in the case of the carcinogenic effects of diethylstilbestrol in humans. This area has been summarized and reviewed by Rice (1984). Warzok *et al*. (1980) found differences in carcinogenicity after transplacental and postnatal administration of drugs, pesticides, or their metabolites. Comparative studies of the carcinogenic activity of procarbazine, methylphenyl nitrosourea, and ethylenethiourea after transplacental and postnatal administration showed marked differences in the frequency and spectrum of tumors induced. For example, administration of procarbazine transplacentally and postnatally resulted in a much higher production of rats with tumors than administration of procarbazine only transplacentally or only postnatally.

Age-related differences in physiology and metabolism probably account for many of the age-related differences in manifestations of toxicity to chemicals. Borghoff and Birnbaum (1985) identified clear age-related changes in glucuronidation and deglucuronidation that depended on the chemical substrate as well as the tissue as a function of age. Also, significant physiologic changes, such as distribution of fat, may account for certain age-related differences in metabolism and toxicity as shown by Yang *et al*. (1984b) in rats dosed for 2 years with ethylenediamine.

The definition of toxicity (Section 1.2) implies that aging is a toxicological phenomenon amounting to accumulation of injury in an organism over short or long periods of time. This accumulation of injury is due to thermodynamics, which means that there are no truly reversible phenomena in nature, even though some processes might get close to it. Therefore, it must be understood that those people who maintain that modern medicine will be capable of prolonging life almost indefinitely are latter day protagonists of some sort of perpetuum mobile, which in terms of science is nonsense. Even replacing organs buys only a short reprieve in old age, because second, third, and subsequent breakdown points are ever closer to each other making the time gained shorter and shorter at exponentially increasing costs.

A toxic insult may be no different in a young than in an old individual if the damage can be recovered from very rapidly (kinetic or dynamic recovery). However, the more irreversible (due to either kinetics or dynamics) an injury is, the larger will be the $c \times t$ contribution to the eventual demise of an organism. Therefore, concern about and protection from exposure of fetuses, neonates, and the young to chemicals with long kinetic or dynamic half-lives is a highly legitimate goal and one of the most important tasks of modern toxicology. However, claiming the need for a 10-fold safety factor for all chemicals to protect this population from significant toxic insult can only originate out from ignorance of the science of toxicology.

Nutrition The relationship between spontaneous or chemically induced carcinogenesis and nutrition has been studied extensively and has been the subject of several reviews (Campbell, 1979; Clayson, 1975; Everett, 1984; Rao *et al*., 1987; and a symposium introduced by Omaye, 1986). However, the subject is beyond the scope of this volume except as it may involve pesticides.

General Nutritional Condition Apparently only extremes of general nutrition have produced observable alterations in the toxicity of pesticides. As reviewed elsewhere (Hayes, 1959), various mammals and even fish are relatively resistant to poisoning by DDT if they are fat rather than thin. The same result has been produced with dieldrin and lindane under experimental conditions (Barnes and Heath, 1964; Geyer *et al*., 1993). Other factors may be involved, but certainly distribution of the insecticide to adipose tissue tends to reduce the concentration of the insecticide at the site of action and thus protecting the organism.

Paired feedings may be used to distinguish those effects of a toxicant secondary to reduced intake of food (Weber *et al*., 1991).

Effect of Starvation If DDT is stored in body fat in sufficient concentration, rapid mobilization of the fat through starvation may lead to poisoning (Fitzhugh and Nelson, 1947). There is an increase in the concentration of poison in the small amount of fat remaining and, by the same token, in all tissues of the body (Dale *et al*., 1962). During mobilization of DDT, excretion is increased by a factor of about 1.4 but the increase is insufficient to prevent poisoning in some rats. Dale and associates pointed out that starvation is unlikely to precipitate poisoning by DDT in humans because even people with heavy occupational exposure to the compound do not store enough of it to produce the effect and because the metabolism of humans is inherently slower than that of rats so that humans cannot starve as quickly.

Some other chlorinated hydrocarbon insecticides are excreted more efficiently than DDT. For example, Heath and Vandekar (1964) found that the average excretion of dieldrin in rats was relatively rapid (5%/day), and that it was more than doubled following a few days of starvation. Therefore, it is not astonishing that it was not possible to precipitate dieldrin poisoning in rats by starving them after they had been fed for 7–18 months at dietary levels up to 15 ppm (Treon and Cleveland, 1955).

The toxicological effect of weight loss associated with infection may be indistinguishable from those associated with starvation (see this Section further below).

Quantity of Dietary Protein In addition to the action involving storage just discussed, nutrition may influence toxicity through metabolism promoted by the liver microsomal enzymes. Murphy and DuBois (1957) showed that male rats maintained on a protein-free diet for 4 weeks had only 24% of the microsomal enzyme activity of normal rats. Also, the liver enzymes of rats that did not receive protein could not be induced by compounds that ordinarily would stimulate these enzymes.

There is great variation in the influence of protein malnutrition on susceptibility to acute poisoning by different compounds. This variation may depend on (a) the net effect of biotransformation, whether detoxification or intoxication (metabolic activation); (b) differences in the relative contribution of biotransformation to toxicity; (c) the ability of some compounds to cause anorexia or other disruption of nutrition; or (d) other mechanisms.

As an example of a difference in the net effect of biotransformation, it may be recalled that the toxicity of aflatoxin, which is detoxified by the liver, is increased by protein deficiency whereas the toxicity of carbon tetrachloride, which is rendered toxic by metabolism, is decreased greatly by protein deficiency (McLean and McLean, 1969).

The LD 50 of DDT and the associated clinicopathological effects showed only slight variation among rats previously maintained for 4 weeks on ordinary laboratory feed and rats fed a synthetic diet containing either normal protein (27% casein) or deficient protein (8% casein) (Boyd and De Castro, 1968). Even after a diet containing no protein, the toxicity of DDT was increased only fourfold (Boyd and Krijnen, 1969b). By extreme contrast, the acute toxicity of captan was increased 2100-fold in rats maintained without protein compared with those fed normal protein (Krijnen and Boyd, 1970). The results for DDT, captan, and a number of other pesticides are summarized in Table 1.10. It may be seen that even a very great increase in protein is without important effect on susceptibility to pesticides. The same is true of a reduction of protein to only one-third of the normal level. However, when protein restriction is severe and especially when it is complete, susceptibility to some pesticides is increased dramatically. It must be recalled that rats that have been maintained without protein for 28 days after weaning, weigh about 30% less than they did when placed on the diet. Two-thirds of these rats die in the first 3 days after their food is withdrawn even though no chemical is administered. It is little wonder that their susceptibility to compounds that cause anorexia is striking.

TABLE 1.10 Estimates of the Increase in the Acute Toxicity of Certain Pesticides in Albino Rats as Related to the Concentration of Protein in Their Diet during 28 Days from Weaning until Dosing[a]

Agent	Percentage in diet					Reference
	0.0	3.5	9.0	26.0	81.0	
Captan	2,100	26.3	1.2	1.0	2.4	Krijnen and Boyd (1970)
Carbaryl	8.6	6.5	1.1	1.0	1.0	Boyd and Krijnen (1969a)
CIPC	8.7	4.0	1.7	1.0	—	Boyd and Carsky (1969)
Diazinon	7.4	1.9	1.8	1.0	2.0	Boyd *et al.* (1969a)
DDT	4.0	2.9	1.5	1.0	3.7	Boyd and Krijnen (1969b)
Endosulfan	20.0	4.3	1.8	1.0	1.0	Boyd *et al.* (1970)
Lindane	12.3	1.9	1.0	1.0	1.8	Boyd *et al.* (1969b)
Monuron	11.5	3.0	1.8	1.0	—	Boyd and Dobos (1969)
Toxaphene	—	3.7		1.0	—	Boyd and Taylor (1971)

[a]*From Boyd et al. (1970), by permission of the American Medical Society.*

Other compounds that have been studied in relation to protein deprivation include carbanolate, parathion, chlordane (Casterline and Williams, 1969), dieldrin (Lee *et al.*, 1964), and TCDD (Muzi *et al.*, 1987).

In a thorough review of diet and toxicity, McLean and McLean (1969) emphasized the opposite effects of protein deficiency on the toxicity of compounds that are detoxified and those that are made toxic by biotransformation, especially in those instances in which the site of biotransformation is also the site of toxic injury. The reviewers also pointed out that reversal of one aspect of deficiency (such as the induction of microsomal enzymes by a foreign compound or by a component of natural diets in animals with borderline protein deficiency) may reverse the entire effect of diet on toxicity. Although there is evidence that malnourished people are unduly susceptible to infection, there is no

clear evidence that the cell's general ability to withstand change and trauma is altered by malnutrition. The relation of nutrition to toxicity must be determined separately for each compound and, under practical conditions, other factors must be taken into account.

Quality of Dietary Protein The acute oral toxicity of heptachlor was found to be 1.6–2.1 times greater in rats that were pair-fed casein than in those that were fed gluten, regardless of whether protein constituted 10 or 18% of the diet. The difference was less or even reversed when the casein diet was fed *ad libitum* and weight gain was greater (Webb and Miranda, 1973). Gluten is an incomplete protein that reduces food intake and permits only an abnormally small increase in body weight of rats that consume it *ad libitum* as their only source of protein. It seems likely that the lower toxicity of heptacholor in rats fed gluten depends on limited conversion of the compound to heptachlor epoxide as a result of limited activity of the microsomal enzymes of the liver. On the other hand, the even greater protection offered by normal intake of high quality protein may result from the presence of normal fat deposits and the sequestering of both heptachlor and its epoxide in fat.

Effects of Fat Dietary fat has been studied less than dietary protein in relation to pesticides. However, Purshottam and Srivastave (1984) found that a high-fat diet significantly protected against mortality from an indirect inhibitor of cholinesterase (parathion) but not from a direct inhibitor (dichlorvos). In contrast, a high fat diet increased the acute toxicity of TCDD compared to high-carbohydrate-fed rats (Muzzi *et al.,* 1987).

Most studies of fat that are potentially of toxicological interest involve carcinogenesis. However, because the findings apply to spontaneous as well as to induced tumors, the relevance to toxicology may be obscure. Briefly, breast and uterine cancer are more frequent in obese women. The possible hormonal basis of this relationship has been discussed (Lipsett, 1983). In fact, several tumors are more common in people who are overweight (Doll and Peto, 1981). An increase in dietary fat-or, in fact, any variable leading to an increase of many spontaneous tumors in experimental animals (Rao *et al*., 1987; Ross *et al*., 1983) – increases the yield of tumors induced by chemicals in experimental animals (Bin *et al*., 1983; Chan and Cohen, 1974; Kollmorgen *et al*., 1981; O'Connor *et al*., 1985; Tannenbaum, 1940). There is some question whether dietary fat has a specific effect or merely contributes calories inasmuch as restricted intake of a particular diet increases longevity and decreases the incidence of tumors (Boissonneault *et al*., 1986; Conybeare, 1980; Rehm *et al*., 1985). In fact, the restriction of diet may be protective even if it is imposed only for several weeks after weaning (Ross and Bras, 1971). On the other hand, for at least some tumors an increase in incidence depends on the specific composition of a fat regardless of its concentration in the diet. Thus, dietary levels of 0.3, 1, or 10% corn oil (which contains linoleate) resulted in more mammary tumors than a dietary level of 10% corn oil from which the linoleate had been eliminated by hydrogenation (Abraham *et al*., 1984). Also, a control group of rats given biweekly 4 ml/kg of corn oil lived significantly longer than *ad libitum*-fed controls with reduced tumor incidence (Rozman *et al*., 2005).

In designing experiments, it is important to note that rats given oil by gavage can have a threefold greater caloric intake than untreated controls (Kraft, 1983).

Miscellaneous Nutritional Effects Deficiency of any essential trace element is injurious in itself. However, a borderline deficiency may predispose to injury by a toxicant. Furthermore, there may be an interaction in the metabolism of trace elements whether essential or not. For example, Brinkman and Miller (1961) found that rats fed molybdenum gained less weight and had lower hemoglobin levels if they were kept in galvanized cages instead of stainless steel cages. Similar effects were produced by increasing the zinc content of the diet of rats fed molybdenum and kept in stainless steel cages.

Isolation and Crowding Either isolation or crowding may influence the behavior, biochemistry, and morphology of animals. Rodents have been most studied in this regard but it seems unlikely that nonrodents are immune. Although very few drugs have been studied in this way, enough work has been done to show that either isolation or crowding has a dramatic effect on the susceptibility of some strains to certain drugs, but little or no effect on their susceptibility to others.

Animals are caged separately in most tests of toxicity. This practice facilitates observation of each animal and permits measurement of individual food intake and collection of individual samples of excreta for analysis. It has been suggested (Hatch *et al*., 1963) that the results of tests on isolated rats do not reflect the functioning of normal animals. It is true that many wild rodents tend to live in small groups and that common laboratory rodents will cluster if permitted to do so. Consideration of isolation and crowding might be crucial in the study of a rodenticide from the standpoint of rodent control. The ultimate objective of most toxicity testing is the safety of humans, not that of rodents. However, in all tests of toxicity, there is a clear need to keep in mind the possible effects of isolation and crowding. Differences in the handling of animals may lead to marked differences in the results of tests in different laboratories or in the same laboratory at different times.

Physiological Effects of Isolation The effects of isolation may depend on sex, strain, and duration of isolation (Wiberg and Grice, 1965). When these factors are held constant, the effects may vary depending on the past grouping history of each animal (Thiessen, 1963). In other words,

isolation changes the susceptibility of animals to crowding. Isolated mice had relatively heavier testes and showed much less locomotion in a standard test than mice held in groups of 5 each. However, when previously isolated mice were placed in groups of 10 each, they showed increased fighting, diminution of testes weight, and a higher level of locomotor activity than mice that had been in groups of 5 before being placed in groups of 10.

According to a review by Hatch *et al*. (1963), isolation of rats or mice for 10 days or less may produce lowered resistance to stress, lower food consumption and weight gain, and smaller adrenals, in comparison with animals held in groups of two or more. Isolation longer than a month may produce the opposite effects, including greater food consumption and a tendency toward larger adrenals. In addition, lower weights of the thyroid, thymus, spleen, and ovary, an increase in oxygen consumption, and absolute leukopenia and eosinopenia have been observed. Other changes have been reported less commonly. The authors interpreted their own findings and those of others as indicating that isolation produces an endocrinopathy probably involving the adrenal cortex.

Effects of Isolation on Susceptibility to Chemicals Isolation may have a marked effect on the reaction of rodents to some chemicals. In other instances, isolation may change the threshold of susceptibility but have little effect on the LD 50 level (Wiberg and Grice, 1965). A very dramatic effect of isolation and its duration on susceptibility to a drug is shown in Fig. 1.20. Not shown by the figure is the fact that rats conditioned by isolation for 3 months remained highly susceptible to isoprenaline even after they had been regrouped for a week (Balazs *et al*., 1962).

Perhaps it is the conditioning produced by isolation rather than isolation itself which is of greatest or most frequent importance in influencing the action of chemicals. It was shown very early (Gunn and Gurd, 1940) that α-amphetamine is more toxic for aggregated than for isolated mice. This result has been confirmed many times. Thus, although the susceptibility of pregrouped mice was increased by grouping as opposed to isolation immediately after dosing (mice died in an average of 53 minutes when grouped, but survived 69 minutes when isolated), the susceptibility of preisolated mice was increased drastically by grouping but was also inherently greater (mice died in an average of 14 minutes when grouped after preisolation but survived 51 minutes when isolated) (Welch and Welch, 1966).

At least in connection with stimulants of the central nervous system, the effects of aggregation may be due to hyperpyrexia associated with increased motor activity resulting from greater response to external stimuli (Peterson and Hardinge, 1967).

It is impossible to discuss here the varied and complex differences in brain and adrenal catecholamines that have been shown to depend on group density. Enough has been said, however, to emphasize the importance of carrying out toxicological experiments under standardized conditions or of varying the conditions knowingly.

Effect of Crowding Crowding is not merely the absence of isolation but a deviation from the norm in the opposite direction. It can cause striking clinical injury and social disintegration, at least in some species. However, there are distinct differences even between rats and mice (Chévedoff *et al*., 1980; Klir *et al*., 1984). The phenomenon has been studied mostly in relation to population control. In a review of this aspect, Christian and Davis (1964) concluded that excess population density leads to increased aggressiveness and other forms of competition and thus (through an endocrine feedback mechanism involving pituitary-adrenocortical

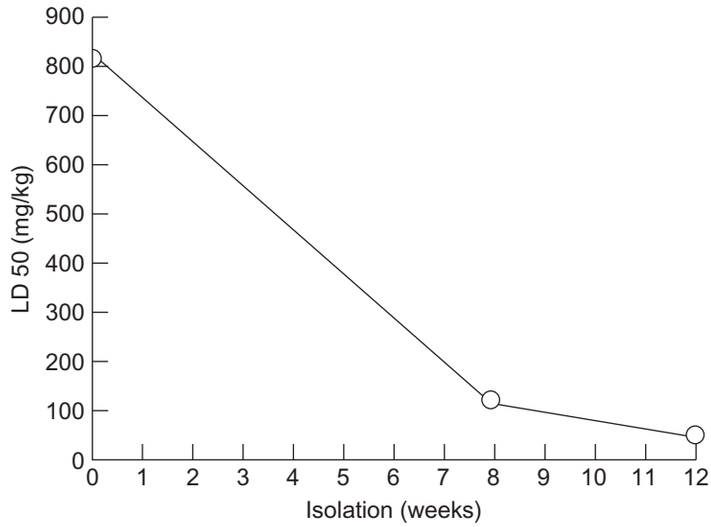

FIGURE 1.20 Effect of isolation on susceptibility of rats to isoproterenol. Based on data from Hatch *et al*. (1963).

activity and inhibition of reproduction) to regulation and limitation of population growth. This mechanism has been demonstrated for some rodents, lagomorphs, and deer, and it may apply to other mammals. According to this concept, other factors such as disease, predation, and weather limit populations occasionally, but the feedback mechanism remains as a safety device to prevent destruction of the environment and consequent extinction.

A tranquilizer can reduce aggression in a population and raise the limit at which the growth curve reaches equilibrium. After three populations of house mice had become crowded and aggression and reproduction had leveled off, chlorpromazine was added to the diet of two of the populations at a concentration of 750 ppm. Although this concentration of chlorpromazine slightly reduced the reproduction of individual pairs of mice tested separately, it decreased aggression and increased breeding success of the crowded mice. Population growth was renewed while the drug was being administered. When chlorpromazine was removed from one of the treated populations, the rate of aggression increased and the number of mice declined. The third population, which served as a control, declined slightly but probably not significantly while the other two increased under the influence of chlorpromazine (Vessey, 1967).

Although crowding (in the sense used in population dynamics) is not likely to occur in a toxicology laboratory, population density undoubtedly has a bearing on the practical use of rodenticides and other poisons to control pests.

Other Social or Psychological Factors Anything that disturbs an animal may influence its physiological reactions and thus possibly change its reactions to foreign chemicals. In some instances, disturbance may have several components and it may be difficult to determine their relative importance. For example, each visit of the investigator or attendant to the animal room involves auditory, visual, olfactory, and sometimes tactile stimuli that animals can detect. Their responses may be unconditioned or conditioned by previous experience. The mere placing of an animal in a cage that differs in shape, area, material, or bedding may influence behavior. Changing the shape of a cage may cause mice to produce wet feces and would interfere with the testing of diuretics or purgatives (d'Arcy, 1962). Chance (1947) reported that by using larger cages he could reduce to about half the toxicity of amphetamine and ephedrine to individually caged mice. Other effects of caging were reviewed by Chance and Mackintosh (1962).

Factors that may be influenced by the location of a cage in the animal room include lighting, temperature, and ventilation. Any influence of the cage itself or of its location is spoken of as a "cage effect." To cancel out such potential effects, investigators may employ random assignment of cages and periodic rotation of their locations. However, at least in regard to tumor incidence, Haseman (1988) reported that among 79 dosed groups of mice the occurrence of apparent cage effects agreed closely with that expected by chance.

Audiogenic seizures are a dramatic, specialized response of some species to certain frequencies and intensities of noise. Approximately 36% of normal Sherman strain rats had seizures in response to intense noise from an electric bell, but the response rose to about 80% in rats receiving dieldrin at a dietary rate of 25 ppm. Although a number of compounds have been studied in this regard, the relationship between response and the tone, intensity, and pulse frequency of sound apparently has received little attention.

Disease Few studies have been made of the relationship between the toxicity of chemicals and the occurrence of disease of other causes. A few exceptions are clearly recognized; for example, silicosis predisposes to tuberculosis. A dosage-response relationship appears to hold, for there is no evidence that inhalation of silica insufficient to cause silicosis has any effect on the occurrence of tuberculosis.

In one instance, a laboratory using pathogen-free rats consistently found higher LD 50 values for a series of test compounds than did other laboratories using normal rats of the same strain in a prearranged study (Weil and Wright, 1967).

Hayes (1982) reported that tube feeding of rats with larvae of *Trichinella spiralis* at the rate of 20 larvae per gram of body weight produced a marked temporary decrease in food intake and a corresponding loss of body weight of about 60 g. The loss occurred over a period of 10 days in rats receiving no other treatment, but continued at a much slower rate for another 6 days in rats that previously had received DDT at a dietary level of 200 ppm (8.5 mg/kg/day for males and 10.5 mg/kg/day for females) for 359 days before infestation with larvae. Biopsy 10 days after infestation showed that the concentration of DDT in body fat had increased from an earlier biopsy average of 1319 $\pm$ 163 (S.E.) ppm to 3105 $\pm$ 1071 ppm as a result of fat mobilization and a partial failure of metabolism and excretion to keep pace with the DDT so mobilized. The surviving rats had recovered fully 38 days later; at that time the DDT concentration in their fat was only 874 $\pm$ 10 ppm, because they had increased their fat stores into which the remaining DDT and that accumulated from continuing dietary intake were distributed. The reduction of food intake and the resulting loss of body weight produced by severe, nonfatal trichinosis were adequate to account for the initial increase in the concentration of DDT-derived material, and the subsequent recovery of weight was adequate to account for the final decrease in the concentration of this material stored in the fat of rats with gastrointestinal phase of this disease. Thus, all of the observed changes could be explained in terms of body weight, as was true for simple food deprivation (see this Section above).

A different and less understood kind of interaction is that in which chemicals appear to reduce resistance to infection or to increase the virulence of an infecting organism.

Inasmuch as some compounds are antibiotic, it is logical that others may be probiotic. However, although some antibiotic reactions have been studied in great detail and are well understood, no careful study has been made of any probiotic interaction although it is well-known that low doses of antibiotics increase body weight gain in many species (Cababrese and Baldwin, 2001a). The antibiotic reactions show dosage-response relationships, and the compounds tend to be specific for groups of microorganisms and sometimes for species or even strains. These same characteristics are likely to characterize any genuine probiotic reactions. Examples of probiosis have been reported, but more investigation is needed to establish their validity and much more work is required to learn whether they are of practical importance and, if so, under what conditions. Although the implications of some individual reports of probiosis seem to have been exaggerated, the broadest theoretical implications have been neglected. In the absence of systematic study, it is impossible to exclude the possibility that probiosis is as important as antibiosis. This would certainly be true if chemical carcinogenesis was, in fact, a form of probiosis by alteration of a virus, a relationship strongly suggested by the work of Price *et al*. (1972), or, more generally, by influencing either a virus or its host, or both, as is now becoming evident for a human cancer caused by the hepatitis B virus (Beasley, 1988).

Reports of probiosis involve polychlorinated biphenyls and duck hepatitis virus in ducks (Friend and Trainer, 1970a); p,p'DDT or dieldrin and the same virus in ducks (Friend and Trainer, 1970b); and lead nitrate and *Salmonella typhimurium* in mice (Hemphill *et al*., 1971).

Enhanced lethality of encephalomyocarditis (EMC) virus infection in suckling mice as a function of topical exposure to a combination of insecticides was reported by Crocker *et al*. (1974). Subsequent studies (Crocker *et al*., 1976) extended these observations to show that the responsible component was the insecticide carrier, the solvent emulsifier system in which the insecticides were prepared. On the contrary, Menna *et al*. (1980) exposed suckling CD1 outbred mice topically to insecticide carrier and found a decreased sensitivity to infection with lethal doses of influenza virus type A/PR8/34 (HONI) compared with untreated or mock-treated control mice. The decreased sensitivity was evidenced by significant increase in mean survival percentage of the mice after inoculation with infectious agent. The decreased sensitivity was virus-dose-related and occurred within a dose range of 2–8 times the LD 50.

One report (Wasserman *et al*., 1969) suggests that any change in response to infection may be complex but by necessity may involve the immune system. Rats given a 200-ppm aqueous suspension of DDT of unstated stability as their only source of water for 35 days not only had heavier livers but slightly heavier adrenals and lighter spleens. The DDT-treated animals showed a rise of serum albumin and some globulin fractions but a decrease of other globulin fractions. Although DDT alone caused a slight increase in the size of the adrenal, it tended to inhibit the greater increase produced by surgery. Whereas the average titer of antibodies to ovalbumin in rats receiving DDT was slightly less than that in controls injected with ovalbumin in the same way, the range of titers in different animals in the same group was so great that the results were difficult to evaluate.

It has not always been possible to confirm reports of a relationship between disease resistance and the intake of a chemical. For example, it has been reported that change in the phagocytic activity of white blood cells is an indication of early intoxication by DDT (Kun'ev, 1965). To test this report, DDT was given to male rats by stomach tube at a rate of 0.25 mg/kg/day for 31 days. Blood taken at intervals from 10 of these rats and from 10 controls was incubated with *Staphylococcus epidermidis.* The proportion of white cells ingesting bacteria and the average number of bacteria ingested per cell were measured. The same measurements were made at intervals on white blood cells from 15 dosed and 15 control rats after they had received bacteria by intracardiac injection. There was no statistical difference in phagocytic activity between the dosed and control rats in either the *in vitro* or the *in vivo* study (Kaliser, 1968).

Temperature The interaction of temperature and the effects of foreign chemicals is complex, but it must be taken into account in the design and interpretation of experiments. Such interaction is most likely to occur in connection with compounds that influence temperature control or metabolic rate, but is not confined to compounds known to have one of these actions. Disturbances and temperature control are more likely to be important in small animals (such as rats and mice) or young animals, simply because their control of body temperature is imperfect at best. No matter what the size of the animals, the investigator should record both the ambient temperature and the body temperature in any study involving temperature as a variable. In some instances, skin temperature or the temperature of the extremities should be recorded because it may be critical but distinctly different from the body or visceral temperature.

For most compounds, minimal toxicity occurs at some temperature between room temperature and thermal neutrality, that is, the temperature at which the animal consumes least oxygen while at rest. In such instances, toxicity increases at temperatures both below and above this point, so that a graph of m ortality or other measure of toxicity against ambient temperature is U-shaped. Examples include ANTU (Meyer and Karel, 1948), parathion (Baetjer and Smith, 1956), warfarin, strychnine, and several common solvents (Keplinger *et al*., 1959). Apparently no compound is known in which the opposite relationship exists, that is, maximal toxicity at some intermediate temperature with lesser toxicity at both lower and higher temperatures.

A smaller number of compounds demonstrate a more or less continuous increase in toxicity corresponding to increasing ambient temperature. In this instance, the graph of toxicity against temperature may be thought of as the right-hand branch of a U-shaped curve. It is often an open question whether the remainder of the curve would be demonstrated if sufficiently lower temperatures were investigated. In any event, continuously increasing toxicity with increasing temperature has been found for dinitrophenol (Fuhrman *et al*., 1943; Keplinger *et al*., 1959) and picrotoxin (Chen *et al*., 1943).

The effects in mice of high and low environmental temperature on the maternal and fetal toxicity of dinoseb and on the disposition of dinoseb were studied by Preache and Gibson (1975). Swiss-Webster female mice dosed with dinoseb were maintained at an increased environmental temperature (32°C) for 24 h or a decreased temperature (0–6°C) for 1.5–4 h. Increased temperature lowered the LD 50 for single injections of dinoseb and reduced temperature had no effect on the LD 50. Exposure of pregnant mice to the increased temperature increased maternal mortality, decreased fetal body weight, and increased the frequency of fetal anomalies relative to animals maintained at normal temperatures. Clearance of dinoseb from plasma or other tissues was not affected by exposure to high or low environmental temperatures.

Very few compounds may be more toxic at lower temperatures so that the graph of toxicity against temperature may be considered the left-hand branch of a U-shaped curve. Whether higher temperatures would complete the curve is often unknown. According to Bogdanovic (1961), picrotoxin is an example but, as already noted, Chen *et al*., (1943) found the opposite in a very careful study.

At least for some compounds that affect body temperature, there are critical ambient temperatures above which compounds cause a rise of body temperature and below which they cause a fall. Different compounds often have different critical temperatures in the same species (Shemano and Nickerson, 1958, 1959). If the change in body temperature is sufficient it may be a major cause of death. Even in the absence of a drug, an ambient temperature of 38°C is lethal to about 50% of mice in 3 h at a relative humidity of 20% (Adolph, 1947). Rats fed malathion at a dietary rate of 4000 ppm died sooner than controls when both were clipped and exposed to an ambient temperature of 1.5°C, but only after their body temperature had fallen to 18°C, which is half of normal body temperature (Marton *et al*., 1962). As Keplinger *et al*. (1959) pointed out, it may be difficult to decide whether the stress of heat or cold renders an animal more susceptible to a compound or whether the compound renders the animal more susceptible to heat or cold. In fact, cold causes reactions of tetanus and hyperresponsiveness of the spinal cord similar in some respects to the reactions caused by strychnine and some other compounds (Koizumi, 1955; Brooks *et al*., 1955).

Many studies of the interaction of temperature and toxicity are carried out in nonacclimated animals. This was true of most of the studies cited in this section. As shown in a paper by Johnson *et al*. (1963) and the associated discussion, acclimatization may alter or even reverse the effect of either heat or cold. For example, Craig pointed out in the discussion that the toxicity of DFP, sarin, and atropine to rats and mice was increased by exposure to cold only if the animals were unacclimatized. This means that the conditions of each study must be stated clearly. It does not mean that investigations of unacclimatized animals are unimportant. People may encounter foreign chemicals at ambient temperatures to which they are not accustomed. The use of hypothermia in medicine is the most dramatic, but perhaps not the most important example.

Temperature can affect absorption, distribution, and also action. As measured by excretion of paranitrophenol, parathion is absorbed more rapidly from human skin at higher ambient temperatures (Funckes *et al*., 1963; Wolfe *et al*., 1970). The maximal average increase in absorption at 40.56°C compared with 14.44°C is apparently on the order of a factor of 4 but may be increased by a factor of 10 or more for the first few hours after exposure (Funckes *et al*., 1963).

Some differences in action at different temperatures may be explained on the basis of dosage at the tissue level, as is true of chlorpromazine (Berti and Cima, 1954). However, this is not always true. For example, the central nervous system depressant norpipanone is about three times more toxic to mice at 29°C than at 18°C ambient temperature (Herr *et al*., 1953). Although the difference is explained at least in part by the fact that, following identical doses, the concentration of the compound in the brain is about 40% greater at the higher temperature, there may be an inherent difference in the reactivity of the tissue. A higher dosage (96 mg/kg) and a resulting higher concentration in the brain (30 ppm) were required to produce the same effect (LD 50) at 18°C than the dosage (33 mg/kg) and brain level (6.7 ppm) required at 29°C (Herr *et al*., 1954). Another example involves the action of DDT on the isolated frog heart. Hoffman and Lendle (1948) found that, in December at a low room temperature, a concentration of 300 ppm was required to produce the same effect that could be produced by 1 ppm or even 0.1 ppm in June at a temperature of at least 22°C. The same compound may produce qualitatively different effects at different temperatures. Fatal doses of chlorpromazine given to mice at an ambient temperature of 38°C cause violent convulsions, but at 13°C they cause prolonged central depression (Berti and Cima, 1955).

The quantitative differences in toxicity associated with temperature are often small but are sometimes dramatic. Cold increased the toxicity of reserpine to unacclimatized mice by a factor of 1200 (LD 50, 0.0 15 mg/kg at 20°C, compared with 18.84 mg/kg at 30°C) (Johnson *et al*., 1963). Cold increased the susceptibility of rats to isoprenaline by factors of about 1,000 in males and 10,000 in females

(Balazs *et al*., 1962). Reviews of temperature effects include papers by Fuhrman and Fuhrman (1961), Ellis (1967), and Weihe (1973). A 2-year study of temperature effects in rats was performed by Yamauchi *et al*. (1981).

In concluding this subsection, it is worthwhile to remind the reader that thermogenesis has been reasonable well-understood, being a result of uncoupling of oxidative phosphorylation potentially in all tissues (White, Hardler and Smith, 1973). Therefore, all uncouplers of oxidative phophorylation will have a temperature effect and vice versa temperature will effect their toxicity. In addition to shivering thermogenesis, Himms-Hagen (1983) distinguished between non-shivering and diet-induced thermogenesis with the former primarily occurring in brown adipose tissue (Himms-Hagen, 1985). 2,3,7,8-Tetra-chlorodibenzr-p-dioxin has been shown to reduce thermogenesis in this tissue (Weber *et al*., 1987). Probably many lipophilic pesticides affect this tissue but thus far little attention was paid to it.

Pressure and Altitude Pressure resulting from altitude may be a factor in the toxicity of any compound, especially one that influences cardiorespiratory function. An example involves the greater toxicities of red squill and digitalis at altitudes higher than most communities (Ward *et al*., 1940). Strychnine is also more toxic at high altitudes (Moore and Ward, 1935). On the other hand, a difference in pressure does not always create a difference in toxicity. Of practical interest is the finding that dichlorvos, at exposure levels far in excess of those proposed for the disinfection of aircraft, exhibited no toxicity to people at 2438 m, a cabin altitude seldom exceeded in normal airline operations of pressurized aircraft (Smith *et al*., 1972).

New problems of toxicology have arisen because submarines and spacecraft may remain out of contact with the atmosphere of the earth for long periods of time. Maintenance of a small closed atmosphere offers a possibility for the accumulation of various gases and vapors that dissipate rapidly in ordinary situations. The toxicity of anything in the small space may be influenced by the fact that the pressure may not be that to which we are accustomed. Elaborate equipment for the study of these problems was first described by Thomas (1965). This issue is not trivial, because continuous exposure above a threshold is more toxic than any other exposure scenario.

Changes in barometric pressure that occur in a laboratory as a part of changes in weather influence the activity of mice (Sprott, 1967) and rats (Olivereau, 1971); increased pressure and sudden falls were associated with increased spontaneous activity, whereas gradual decreases in pressure had the opposite effect.

It is well known from physics that all phenomena of nature are temperature-dependent unless the change in temperature is so small that its effect is not measurable. The same can be said for pressure and volume except for a few limiting conditions. For the most part these variables play a minor role in toxicology for obvious reasons. Most laboratory animals are homeotherms, which means that biological processes take place at almost constant temperature. Changes in ambient pressure are also comparatively small and very few species change their volume dramatically. However, under extreme conditions (hibernators, high altitude, some frogs and toads) the dependence of $c \times t$ on these variables could possibly be established. Therefore, it should be noted that we are aware of the fact that the fundamental law of toxicology as formulated in this chapter should be written as $(c \times t = k)_{T,p,v}$ or $(c \times t = k \times W)_{T,p,v}$.

Light and Other Radiation Although this section is concerned with the biological effects of light on toxicity, it should be recalled that some compounds undergo chemical change when exposed to radiation. Some of these changes have been demonstrated in pesticides and others may occur. Reactions in the upper atmosphere are considered important in degrading a variety of airborne compounds.

Some pesticides known to be susceptible to photodynamic action include p,p'-DDT (Roburn, 1963), p,p'-DDE (Roburn, 1963), p,p'-DDD (Roburn, 1963), aldrin (Roburn, 1963), dieldrin (Robinson *et al*., 1966; Roburn, 1963). and endrin (Roburn, 1963). It has not been proved that these purely physicochemical changes are of any practical importance in the toxicity of any pesticide. There is some evidence that poisoning of crop workers may result from residues of paraoxon in fields treated with parathion (Milby *et al*., 1964). Conversion of parathion to paraoxon and other derivatives has been demonstrated in the laboratory (Frawley *et al*., 1958; Payton, 1953). However, it is not clear what factors favor the production and persistence of enough paraoxon in the field to produce poisoning.

An old but still useful review of photodynamic action and diseases caused by light is that of Blum (1941). Weihe (1976) thoroughly reviewed the effect of light on laboratory animals. The need for standardized lighting in animal rooms is well documented (Kaitz and Auerbach, 1979; Reiter, 1973; Robinson and Kuwabara, 1976; Weisse *et al*., 1974).

Ionizing Radiation The biological effects produced by X-rays and other ionizing radiation have been studied extensively. A description of these effects is beyond the scope of this volume, even though gamma rays from radioactive cobalt were used to sterilize screw-worm flies to eradicate this destructive species in the southeastern United States.

A useful review of the biological effects of ionizing radiation is that of Schwan and Piersol (1954, 1955).

Ultraviolet Radiation In addition to the direct photochemical action mentioned at the beginning of this section, ultraviolet radiation with a wavelength in the range of 0.29–0.32 μm is responsible for sunburn. Ultraviolet light

also produces "farmer's skin," an increased incidence of skin cancer, and the conversion of 7-dehydrocholesterol or a similar precursor in skin to vitamin D. These effects and the general action of sunlight were reviewed in detail by Blum (1945).

Visible Radiation Difference in the intensity and wavelength of light within the visible range may influence the production of a variety of physiological effects which may interact with the effects of toxic substances or even become manifest only in the presence of such substances. Kueter and Ott (1964) reported acceleration of the appearance of carcinoma, increased aggressiveness, and reversal of the sex ratio as effects of artificial light from various commonly used sources.

Animals in toxicology laboratories are routinely housed in cage racks with multiple shelves to maximize the use of animal room space. Greenman *et al*. (1982) reported the influence of shelf level on retinal atrophy in mice. It had been shown earlier (Noell *et al*., 1966) that 24-h exposure of unrestrained rats to light from ordinary fluorescent bulbs causes irreversible damage.

Greenman *et al*. (1984) evaluated the association between cage shelf level and spontaneous and induced neoplasms in about 21,000 mice being used in a study to evaluate the carcinogenicity of 2-acetylaminofluorene. There was evidence for a shelf level influence on five of the six major spontaneous neoplasms noted. Time to onset of uterine polyps and reticular cell sarcomas was significantly delayed on the top shelf of five of six animal rooms. Also, there was significant delay in the onset of lymphomas, adrenocorticoadenomas, and lung alveolar cell tumors on the top shelf when data were combined from all six animal rooms, but these delays on the top shelf were significant in no more than two of the six animal rooms when rooms were analyzed separately. Thus, this study provided evidence that a shelf level must be considered in the design and analysis of carcinogenesis studies. In contrast to these observations, Haseman (1988) reported a lack of cage effect on liver tumor incidence in B6C3F$_1$ mice constituting a total of 89 dosed groups showing increased liver tumor incidence.

Experimental design protocols that include random assignment of columns of cages to dosed and control animals, periodic rotation of cage location, and individual caging of animals reduced the likelihood that differences in lighting or other factors associated with cage placement of animals could influence the results of toxicity studies.

Photoperiodicity Photoperiodicity of visible light determines or synchronizes circadian rhythms and, in combination with changes in temperature, is responsible for seasonal changes in physiology (see Section 1.5.7.1).

Photosensitization Some chemicals make cells more susceptible to the action of light, especially ultraviolet light. Effects have been reported to result from wavelengths ranging from 0.29 to 0.50 μm (Daniels, 1965). Most compounds with this property are fluorescent (Blum, 1941), Although photosensitization usually affects the skin of vertebrates, other tissues are not immune. For example, the perfused turtle heart was arrested by a porphyrin preparation when exposed to light, but not in the dark. The reaction was not caused by a diffusible toxin. A second heart perfused in the dark with perfusate from the first heart was not affected (Rask and Howell, 1928). Actions on other vertebrate tissues as well as free-living cells, viruses, and proteins including enzymes have been demonstrated (Blum, 1941).

One of the outstanding characteristics of photodynamic processes is that they occur only in the presence of molecular oxygen. However, photodynamic uptake of oxygen differs strikingly from normal oxidative metabolism in regard to respiratory quotient and sensitivity to heat and chemical inhibitors (Blum, 1941).

Whereas chemical photosensitization generally is activated by ultraviolet light, visible light may be an activator also, at least in some organisms. For example, although paramecia are not injured or sensitized to heat by visible light of high intensity, they readily are killed by this light in the presence of photodynamic dyes, and they are sensitized to heat by sublethal dosages of light. Cells so sensitized are killed when subjected to otherwise harmless temperatures. If the light and heat are applied in the reverse order, no ill effects are observed (Giese and Crossman, 1946).

In most instances, the biochemical basis of photosensitization is not understood, but it certainly can involve basic components of protoplasm. Deoxyribonucleic acid suspensions become less viscous when irradiated *in vitro* in the presence of eosin, methylene blue, 1,2-benzanthracene or 20-methylcholanthrene. It is thought that the reaction involves depolymerization (Koffler and Markert, 1951).

Photosensitization has been caused in one species or another by a wide range of compounds. In addition to the porphyrins, the following materials have caused some degree of photosensitization in humans: methylene blue, many phenothiazine compounds, many furocoumarin compounds, anthracene and acridine derivatives, 5-methoxypsoralen (the active principal of oil of bergamot used in perfumes) and related materials from other plants, griseofulvin, demethylchlorotetracycline and some other antibiotics, sulfonamides and their derivatives (including some oral hypoglycemic agents), bithionol, hexachlorophene, and other miscellaneous drugs. Note that many of these compounds consist of three aromatic or heterocyclic rings in a linear configuration. Substitution with sulfur or nitrogen may lead to an increase in photosensitizing capacity (Daniels, 1965).

A number of pesticides have chemical structures suggesting they might act as photosensitizers. This property has been observed in connection with oxythioquinox, phenothiazine and griseofulvin.

Porphyrins are probably the cause of more frequent and more serious photosensitization in humans than all other materials combined. However, photosensitization does not occur in all cases in which the concentration of porphyrins in the blood and excreta is increased. It is important to note that the chemical most effective for disturbing this metabolism is a now banned pesticide, hexachlorobenzene. Detailed reviews have been written by DeMatteis (1967), Schmid (1966), and Jaffe (1968), and Courtney (1979).

Circadian and Other Rhythms A wide range of biological rhythms and their bases have been reviewed at length (Sollberger, 1965). The word "circadian," from the Latin *circa* (about) and *dies* (a day), refers to the rhythmic repetition of certain phenomena in living organisms at about the same time each day.

Of course, some circadian rhythms have been common knowledge for centuries. Some animals are nocturnal and others are diurnal. One might suppose that this difference in activity pattern depended merely on species differences regarding direct response to light. Experimentation has revealed that the situation is often not so simple. The activity pattern may persist, with or without modification, when an animal is placed in continuous darkness or continuous light. Even when experimentally imposed conditions of light change the pattern, the regulation may be temporary and the natural rhythm may eventually exert itself. For example, although Siegel (1961) found that the diurnal feeding pattern of rats disappeared in 6–10 days in rats transferred to continuous light, Wiepkema (1966) found that mice reared for two generations in continuous light showed a marked circadian rhythm in their feeding.

The persistence of circadian rhythm in the absence of any known external clue to the passage of time is one kind of evidence that has led to the hypothesis of the "physiological clock," Although the anatomical location and mode of action of such a clock is not known precisely, there is convincing evidence that some circadian rhythms are endogenous. Both endogenous and exogenous circadian rhythms are adjusted and regulated by photoperiod.

Under natural conditions, photoperiodicity depends on the movement of the earth. Rotation of the earth produces succession of day and night. Revolution of the earth around the sun produces the seasons. In the temperate and arctic zones, the days are longer in summer and shorter in winter. Furthermore, the difference in the length of daylight at these seasons increases progressively from the equator to the poles. Consequently, during spring and fall, the rate of change in the relative duration of light and darkness is greatest at the poles and least near the equator. The ability of organisms to use photoperiodic cycles as clues to impending seasonal change implies that they possess the ability to distinguish between long and short lengths of daylight. This ability is one factor in the complex adaptation of organisms to their environment.

The complicated and varied effects of photoperiodicity on organisms have been abundantly demonstrated by experiments designed for that purpose. There is a danger that the possible importance of photoperiodicity will be forgotten in experiments designed for other reasons. Many modern animal rooms have only artificial lighting, but the lighting cycle and the adaptation of the animals to it frequently are not mentioned in descriptions of methods used in toxicological studies.

It appears that the effects of circadian rhythms and photoperiodicity in invertebrates, especially insects, have been studied more thoroughly than those effects in mammals. Valuable reviews of the physiology and ecology of photoperiodism in insects have been written by Beck (1963) and Danilevskii (1965). It is impossible to go into the matter in detail here. It is pertinent to record that one or more species of insects or mites show definite diurnal variation in their susceptibility to some pesticides, including dichlorvos (Polcik *et al*., 1964), methyl parathion (Cole and Adkisson, 1964), DDT (Beck, 1963), and potassium cyanide (Beck, 1963).

Probably some of the information on invertebrates would be of value in connection with studies in mammals. However, it is already clear that circadian rhythms are important in a number of physiological functions of mammals, including their susceptibility to some poisons.

Circadian Rhythms in Mammals In mammals, as in insects**,** endocrine functions may be influenced directly or otherwise by light and may involve circadian rhythms. For example, hepatic tryptophan pyrrolase and its circulating substrate, whole-blood tryptophan, have a circadian rhythm in mice that is practically eliminated by adrenalectomy (Rapoport *et al*., 1966). However not all liver enzymes are so greatly influenced by adrenalectomy. Civen *et al*. (1967) showed that the rhythmicity of tyrosine ketoglutarate transaminase (TKT) is little altered after adrenalectomy.

The same authors Civen *et al*. (1967) noted that TKT is rapidly induced by various agents but that phenylalanine pyruvate transaminase (PPT) is not induced during the same time period and does not show circadian variation. On the basis of this and some other evidence, they suggested that the sensitivity of an enzyme's regulating system to inducing agents may be related to the inherent circadian rhythm of the enzyme.

The exact function, if any, of the pineal gland (epiphysis) is still in doubt. Because of its histology and the nature of its embryonic origin, it has been suspected for a long time that the structure has an endocrine function. This possibility, with special reference to neurohormonal control, seems to gain support from the demonstration (Quay and Halevy, 1962) that the pineal gland is rich in serotonin. Studies of the gland illustrate the complex interrelation that circadian rhythms may show in one small detail of mammalian physiology. In the rat, the serotonin content of

the gland shows a circadian rhythm (Quay, 1963; Snyder *et al*., 1965) which is somewhat modified by the estrus cycle (Quay, 1963). The rhythm persists in rats kept in the dark or in rats whose eyes have been removed, provided the animals are otherwise intact. The rhythm is abolished in intact rats by continuous light and also abolished by interruption of sympathetic innervation (Fiske, 1964; Snyder *et al*., 1965). The rhythm is changed in a matter of hours by change in photoperiods (Quay, 1963). The rhythm is not affected by removal of the pituitary, thyroid, adrenals, or ovaries (Snyder *et al*., 1965). The rat pineal gland also shows circadian rhythms for hydroxyindole O-methyltransferase (HIOMT) (Axelrod *et al*., 1965) and endogenous melatonin (Quay, 1964). However, these rhythms are opposite in phase to that of serotonin and also differ in that they do not persist in animals kept in the dark. The rhythms for HIOMT and melatonin are directly responsive to light. All three rhythms (HIOMT, melatonin, and serotonin) are interrupted by removal of the superior cervical ganglia (Fiske, 1964; Snyder *et al*., 1965; Wurtman *et al*., 1964). Because the nerve pathways are probably noradrenergic, McGeer and McGeer (1966) explored the possibility that there might be a circadian rhythm in the ability of the nerve endings of the pineal gland to form noradrenalin. They found such a rhythm in the activity of tyrosine hydroxylase, the rate-controlling enzyme in the synthesis of noradrenalin.

Some circadian rhythms involve reactions known to be of fundamental physiological importance. For example, Spoor and Jackson (1966) showed that the beating rate of rat atria decreased more in response to a standard concentration of acetylcholine if they were isolated at 1100 hours than if isolated at 2300 hours. The food intake of rats normally follows a circadian rhythm, the details and modification of which were studied by Siegel (1961).

The preceding examples involve rhythms with a single major peak and a single major trough during each 24-h day. The rhythm is either a direct response to the periodicity of light or, if endogenous, at least is synchronized by light. Lindsay and Kullman (1966) reported that the survival time of female mice given a standard dose of sodium pentobarbital varied during a 12-h period in such a way that the graph showed several inflections. Although this result is unexplained and unconfirmed, it is not unique. A similar result was reported for the susceptibility of boll weevils to methyl parathion (Cole and Adkisson, 1964). The weevils showed greatest resistance at the beginning of the light period no matter whether it started at 0600 hours (14-h photophase), 0700 hours (12-h photophase), or 0900 hours (10-h photophase). Regardless of the length of the photophase, peaks of resistance recurred at intervals of about 6 h with intervening troughs of susceptibility. Why the 3-h cycle corresponded with the sampling interval was unexplained. In any event, it is interesting that both examples of multiple peaks involve susceptibility to a foreign compound. Nothing is known of the enzymatic or other physiological basis for the reported phenomenon.

Typical circadian rhythms (one peak and one trough during a 24-h day) are involved in the responses of several mammals to a number of foreign chemicals. Such cycles of susceptibility were observed at least as early as 1949 (Carlsson and Serin, 1950). As reviewed by Sollberger (1965), a 24-h rhythm in sensitivity of mammals to a number of drugs has been reported. Compounds involved include insulin, hormones, narcotics, sedatives, tranquilizers, bacterial toxins, and carcinogens. Other examples include lidocaine (Lutsch and Morris, 1967), methopyrapone (Ertel *et al*., 1964), nikethamide (Carlsson and Serin, 1950), and pentobarbital (Davis, 1962).

Human circadian rhythms can persist in continuous darkness; social cues are sufficient to entrain them (Aschoff *et al*., 1971).

It is clear that circadian rhythms or the effects of light periodicity should be considered when there are unexplained differences in the results of different laboratories or in the results of the same laboratory at different times.

Examples of the influence of pesticides on the circadian rhythms or the effect of circadian rhythms on the toxicity of pesticides are not common, but Nicolau (1982) reported on the effects of pesticides on the circadian time regarding the structure of the thyroid, adrenal, and the testis in rats. Four herbicides, a fungicide, and two insecticides were tested. A wide variety of rhythm alterations was found. There was significant desynchronization of thyroid and adrenal gland functions. In contrast, there was almost no effect on the rhythms in the testis. Nicolau (1983) also reported on the effect of dichlorvos and trichlorfon on circadian rhythms of RNA, DNA, and protein synthesis in the rat thyroid, adrenal, and testis during exposure to these chemicals for 90 days. Prolonged exposure of Wistar rats to trichlorfon at a concentration of 10 ppm in the diet led to marked changes in the circadian rhythm of the thyroid DNA and protein and adrenal DNA content, phase alterations in thyroid RNA and adrenal DNA rhythms, and marked decrease in the amplitude of the adrenal DNA and protein rhythms. Exposure to dichiorvos in the diet at a concentration of 5 ppm led to phase alterations, without a change in the time-qualified mean, of the circadian rhythms in DNA, RNA, and protein content and marked decrease in amplitude of the DNA rhythm in the thyroid and adrenal. There were no alterations in the rhythms of testicular function with either of the pesticides studied.

Other Factors Influencing Toxicity Undoubtedly, many factors in addition to those discussed in the preceding sections may influence toxicity under certain circumstances. However, these other factors are probably not of major importance in mammalian toxicology.

Seasonal Differences Seasonal differences are of tremendous importance in the physiology of cold-blooded animals and in their responses to toxicants. Presumably, similar differences would hold for mammals that hibernate, but the question has received little attention. Seasonal variation in the LD 50 of mice treated with organic solvents has been reported (Wolff, 1985), but Gaines and Linder (1986) found no *seasonal* pattern for either parathion or DDT although the bimonthly *saw-toothed* pattern for DDT was the same for male and female rats.

Relative Humidity Presumably, relative humidity might influence the reaction of an animal to a toxin in any situation in which humidity was already critical for the animal's health, for example, maintenance of normal body temperature in a hot environment. Such an interaction would seem most likely in connection with compounds that increase heat production or influence temperature control. However, no instance of such an effect on toxicity seems to have been reported. What has been reported is an effect of relative humidity on the absorption of insecticides and, therefore, on their availability for evaporation or absorption from surfaces.

It has been shown that parathion is absorbed more rapidly by the human skin at higher temperatures (Funckes *et al*., 1963; Wolfe *et al*., 1970). What part humidity (especially from sweat) may play in the process apparently is not known. It is probable that mammals may be able to absorb pesticides from their skin surfaces more readily if the surface is moist, because absorption is usually increased after application of occlusive patches.

Aquatic Factors Because it is sometimes suggested that fish or other aquatic organisms be used for bioassay of toxicants that might influence mammals, it is necessary to record that the welfare of aquatic organisms is influenced by several environmental factors that have little meaning for land animals. Most important is continuous exposure in an aquarium.

pH The influence of pH on toxicity often is explained easily in terms of the availability of toxicant. Toxic ions or alkaloids may be much more soluble or easily absorbable at one pH than at another.

Water Hardness Henderson and Pickering (1957) found that water hardness had a significant effect on the toxicity of trichlorfon to fathead minnows but no significant effect on the toxicity of nine other organic phosphorus compounds they studied. Water hardness had little or no effect on the toxicity of chlorinated hydrocarbon insecticides to fish (Henderson *et al*., 1959).

Chlorine Content Ordinary tap water may contain enough free chlorine to kill some fish. This must be kept in mind in bioassays on fish.

It must be pointed out that aquatic toxicity is kinetically similar to continuous inhalation exposure. Therefore, toxicity determined in an aquarium in a species that spends all its time in water will strictly obey Haber's rule, unless the experiment harbors some uncontrolled variables discussed by Hayes in preceding subsections.

1.5.7.2 Species Differences Due to Kinetics

Kinetics (*K*) is the mathematical description of the time course of a chemical in an organism as affected by absorption, distribution, biotransformation, and excretion. It should be emphasized that species differences in *K* may be due to any of these processes. A widely held reductionist viewpoint led to only biotransformation being given due scrutiny as a potential cause of species differences in the disposition of xenobiotics (Caldwell, 1982). However, an astute investigator ought to embark upon studying the disposition of a new compound without such bias. The following discussion will demonstrate the importance of bio-transformation for species differences, but it will also point out the pitfalls of failing to take into consideration other important processes involved in the disposition of xenobiotics.

Absorption

Gastrointestinal Absorption In general, gastrointestinal absorption of xenobiotics was thought to be similar between species. The work of Dreyfuss and colleagues illustrates the fallacy of this assumption (Dreyfuss *et al*., 1978). Absorption of nadolol [calculated from AUC after intraperitoneal (ip), intravenous (iv), and oral (po) dosing] was essentially complete in the dog, substantially less in humans, and quite limited in the rat (Table 1.11). Urinary and fecal excretion of nadolol support the bioavailability data. However, excretory data further indicate that, in addition to the nonabsorbed portion of this compound, biliary and possibly nonbiliary sources also contribute to the fecal excretion of

TABLE 1.11 Absorption and Excretion of Radioactivity in Rats, Dogs and Humans after Nadolol Dosages[a]

Species	Dose (mg/kg)	Route	Percent of dose excreted		Percentage of dose absorbed
			Urine	Feces	
Rat	20	po	11	84	18
	20	ip	62	31	(100)
Dog	25	po	76	28	102
	25	ip	75	12	(100)
Human	2	po	25	77	34
	2	ip	73	23	(100)

[a]*Modified from Dreyfuss et al. (1978).*

this compound. Calabrese (1984) reported evidence for species differences in the absorption of at least 38 compounds, indicating that nadolol may not be an exceptional case. It is more likely that the possibility of species differences due to differences in absorption has seldom been considered or examined. This is surprising because numerous characteristics of the gut suggest that species differences in the absorption of xenobiotics may be expected.

The rate-limiting barrier in the absorption of most xenobiotics is the unstirred water layer along the intestinal mucosa (Hayton, 1980). The effect of the unstirred water layer as a possible cause of species differences in absorption of xenobiotics has not been investigated. However, Thomson *et al*., (1983) studied the effect of the unstirred water layer on the absorption of fatty acids and cholesterol. These authors concluded that the thickness of the unstirred water layer may contribute to species differences in the absorption of lipophilic compounds, but other tissue-specific differences must also exist because species differences persisted when the unstirred water layer was diminished by stirring. Based on these considerations, it is reasonable to assume that the permeability of the gut for xenobiotics transported by passive diffusion can be species-dependent.

Anatomical (allometric) considerations are another likely reason for species differences in intestinal absorption. The relative length of intestinal segments is quite variable (Iatropoulos *et al.,* 1986), and substantial functional differences exist between such species as ruminants and omnivores (Smith, 1986). Because most xenobiotics are transported across the gastrointestinal mucosa by passive diffusion, and because this transport is surface-area- and site-dependent, it can be expected that these factors will be responsible for species differences in some in-stances. Many xenobiotics are weak organic acids or bases. For such compounds, gastrointestinal absorption is dependent on the pH along the gastrointestinal tract. Table 1.12, modified from the work of Smith (1986), shows that each segment of the gut reveals considerable species specificity, with differences of up to two pH units. This translates into two orders of magnitude difference in terms of the concentration of the undissociated versus dissociated moiety of a weak organic acid or base. Obvious consequences of such differences for absorption have been discussed by Shanker (1962).

An additional factor that may result in species-dependent absorption of xenobiotics is the gastrointestinal flora. In general, the microflora of animals is remarkably similar, although qualitative and quantitative differences have been reported (Smith, 1965). Notable deviations to this generalization do exist, such as the rabbit and humans (Table 1.13). In contrast to other species, the microflora in these two species is essentially absent in the upper gastrointestinal tract. Because absorption of some xenobiotics requires prior bacterial hydrolysis, some species differences may be due to differences in microflora. The example of cycasin is discussed by Rozman and Iatropoulos (1989). Cycasin is poorly absorbed by gnotobiotic animals; however, the aglycon of cycasin is readily absorbed. Therefore, species with bacterial β-glucosidase activity in the upper small intestine readily absorb the aglycon (methylazoxymethanol), but species like humans, with very low levels of microflora in the upper gastrointestinal tract, may not absorb this compound to any major extent.

Dermal Absorption Species differences related to dermal absorption of xenobiotics have been more appreciated (Calabrese, 1984). Dermal absorption of endogenous or exogenous compounds may vary by orders of magnitude (Kao *et al*., 1985). According to Dugard (1983), two factors are important in dermal absorption of chemicals: the appendages (sweat ducts, pilosebaceous ducts) in the early phase of absorption and the stratum corneum in the late and dominating phase of absorption. Both factors are highly species-dependent. Because the stratum corneum is much thicker in humans than in animals, human skin is usually less permeable for xenobiotics than is animal skin. However, the thinner stratum comeum in animals is often compensated for by a relatively thick hair cover, diminishing direct contact of the skin with a xenobiotic. Sweat and pilosebaceous ducts also reveal great species variability.

TABLE 1.12 pH of the Gastrointestinal Contents of Various Species[a]

Species	pH				
	Stomach	Jejunum	Cecum	Colon	Feces
Monkey	2.8	6.0	5.0	5.1	5.5
Dog	3.4	6.6	6.4	6.5	6.2
Rat	3.8	6.8	6.8	6.6	6.9
Rabbit	1.9	7.5	6.6	7.2	7.2

[a]*Modified from Smith (1965).*

TABLE 1.13 Number of Microbes and Their Distribution along the Gastrointestinal Tract of Various Species[a]

Species	Stomach	Jejunum	Colon	Feces
Monkey	23	24	41	38
Dog	19	20	40	43
Rat	18	23	37	38
Rabbit	4	5	13	13
Human	2	4	10	—

[a]*Modified from Smith (1965) and Hallikainen and Salminen (1986). Expressed as $\log_{10}$ of viable counts.*

Eccrine sweat glands are located in the pads of the extremities of all mammals. However, the general body surface of humans contains 100–600/m^2 of coiled tubular sweat glands, whereas rodents and rabbits have none (Szabo, 1962). The number of pilosebaceous ducts in humans and pigs is similar (about 40/cm^2), but rodents may have 100 times more (Calabrese, 1984). Moreover, biotransformations in skin that facilitate absorption also display great species variability (Kao *et al*., 1985).

Another important potential rate-limiting step in the dermal absorption of chemicals is the cutaneous blood flow. Due to an important thermoregulatory function of the skin in humans as opposed to furred animals, there is a much more extensive vasculature in humans than in most mammals (Calabrese, 1984). This brief discussion illustrates that species differences in the disposition of xenobiotics after dermal exposure may be due to numerous anatomical, physiological, and biochemical factors.

Distribution This process in the disposition of xenobiotics has rarely been considered as a potential cause of inter-species variability. However, a closer scrutiny of the literature indicates that this may be an unjustified assumption.

Plasma Protein Binding The disposition of clofibrate (clofibric acid) is a case in point. Plasma protein binding of clofibric acid reveals considerable species differences between mice, rats, and humans, which roughly correlates with the half-lives of this compound in these species (Table 1.14). Because clofibric acid is primarily eliminated in all three species by glomerular filtration without tubular reabsorption (pK = 3), differences in the free fraction of this compound in plasma of various species are likely to contribute greatly to the observed species differences. The other major factor is renal clearance (blood flow dependent).

Additional factors that influence plasma protein binding may also be responsible for species differences, as discussed by Wilkinson (1983). Most important are species differences in the concentration of albumin, binding affinity, and competitive binding of endogenous substances.

Tissue Binding This is an area where information is scarce. Kurz and Fichtl (1983) reported good correlation for the binding of drugs to muscle of man and rabbit. However, a more typical example is reported by Batra *et al*. (1986), which shows that interspecies variations in the tissue distribution of mitoxantrone are unpredictable and may vary by more than an order of magnitude.

One frequently overlooked cause of species differences in the distribution of xenobiotics with large tissue accumulation tendency (e.g., storage in fat) is the different rate of growth of mammals (Scheufler and Rozman, 1984). As Freeman *et al*. (1989) demonstrated using a physiologically based pharmacokinetic model, tissue and whole body growth contribute more to the distribution profile of hexachlorobenzene than does excretion.

Biotransformation This is the best-documented cause for species differences in the disposition of xenobiotics (Caldwell, 1981, 1982). Very informative in this context is Walker's (1980) compilation of monooxygenase activities in 65 vertebrate species. The presence of cytochrome P450 and its associated electron transfer components across broad taxonomic classes suggests that this system has arisen from some ancient genome. The most likely explanation for the vast species differences in the expression of this genome is the evolutionary need to respond to changing diet, life style, and habitat.

Phase I Biotransformations Caldwell (1981) illustrates the consequences of species differences in phase I biotransformation for the disposition of a number of amphetamines. Deamination is the major pathway of amphetamine biotransformation in rabbits and guinea pigs, whereas aromatic hydroxylation is the predominant route of biotransformation in the rat. The rhesus monkey utilized both pathways to a similar extent, and the marmoset neither one. Correspondingly, the marmoset excreted an administered dose unchanged, whereas the other species eliminated little of the parent compound, but rather the respective metabolites. The broad tissue (liver and intestine) substrate specificity of monooxygenase isozymes is shown in Table 1.15. This table also illustrates the evolutionary importance

TABLE 1.14 Plasma Protein Binding and Half-Life of Clofibric Acid in the Mouse, Rat, and Human[a]

Species	Plasma protein binding (%)	Half-life (h)
Man	97	21
Rat	75	6
Mouse	45	2

[a]Modified from Cayen (1980).

TABLE 1.15 Species Differences in Substrate Specificity of Monooxygenases in the Liver and Intestine[a]

Species	Benzo[*a*]pyrene hydroxylase[b]		Ethylmorphine *N*-demethylase[b]	
	Liver	Intestine	Liver	Intestine
Rat	0.33	0.14	3.8	ND
Mouse	0.15	0.10	3.4	ND
Rabbit	0.06	0.84	1.3	11.2
Guinea pig	0.07	0.37	1.4	8.8

[a]Modified from Gregus et al. (1983) and Laitinen and Watkins (1986).
[b]Expressed as nmol/min/mg protein; ND, not detectable.

TABLE 1.16 Effect of Diet on Phase I Biotransformations in the Gut[a]

Dietary condition Fat deficiency	Phase I biotransformation Decreased
Fat excess	Decreased
Cholesterol excess	Increased
Cholesterol deficiency	Decreased
Copper excess	Increased
Selenium deficiency	Decreased
Cabbage	Increased
Brussels sprouts	Increased

[a]*Modified from Laitinen and Watkins (1986).*

of diet in the development of monooxygenase activities, because rats and mice, rabbits and guinea pigs are similar, respectively, but omnivores differ greatly from herbivores. Composition of the diet may also play an important role in the biotransformation of xenobiotics on a day-to-day basis. Table 1.16 demonstrates that certain phase I biotransformations may be increased or decreased depending on dietary factors. It is thought that dietary components induce or inhibit monooxygenases and thereby alter phase I biotransformation of xenobiotics with corresponding consequences of increased or reduced rates of elimination.

Phase II Biotransformations In the disposition of xenobiotics, phase II biotransformations are usually more important than phase I reactions, although phase I reactions may be a prerequisite for subsequent phase II biotransformations in some instances. For example, insertion of a hydroxyl group or epoxidation (phase I biotransformations) does not change the lipophilicity (and hence reabsorbability) of a xenobiotic to any great extent. However, phase II biotransformations (glucuronidation, sulfation, glutathione conjugation) increase water solubility very substantially, and hence increase the renal or biliary excretability of xenobiotic metabolites. The same factors affect phase II biotransformations that affect phase I biotransformations. Therefore, it is not surprising that vast species differences exist in the phase II enzyme-dependent disposition of even structurally highly related xenobiotics (Table 1.17). Thus, accurate species-to-species predictions regarding phase II biotransformation-dependent disposition of xenobiotics remain elusive, which may or more often may not hamper the predictive value of one species for another in terms of toxicity. The crucial role of genetics in enzyme activities is clearly illustrated by Table 1.18, showing that phylogenetic relationships allow at least some generalizations.

Excretion This is the final and irreversible step in the disposition of xenobiotics. Consequently, any of the previous

TABLE 1.17 Urinary Excretion of Phase II Biotransformation Products of Organic Acids[a]

Species	Naphthylacetic acid[b]		Hydratropic acid[b]	
	Amino acid conjugates	Glucuronides	Amino acid conjugates	Glucuronides
Rat	23	51	0	64
Ferret	72	19	91	5
Rhesus monkey	3	83	0	75

[a]*Modified from Symchowicz et al. (1967).*
[b]*Expressed as percentage of dose; ND, not determined.*

TABLE 1.18 Predictive Pattern of Animal Biotransformation Reactions for Humans[a]

Species	Prediction	
	Good or fair[b]	Poor or invalid[b]
Rat	41	59
Other nonprimate	59	41
Rhesus monkey	92	8

[a]*Modified from Caldwell (1981).*
[b]*Expressed in terms of % of occasions predictable for humans in each category.*

steps (absorption, distribution, biotransformation), as well as differences in excretion itself, may be responsible for species differences in the elimination of xenobiotics. In a simple case (e.g., inulin), when a compound is injected intravenously (no absorption) and does not bind to plasma protein, or does not distribute to tissues, or does not get biotransformed, and its only route of elimination is glomerular filtration, then the cause of species differences can be attributed solly to the rate of blood flow to the kidneys. However, in most instances the situation is much more complicated, as discussed previously for the individual steps in the disposition of xenobiotics. A few important examples follow that will illustrate the major factors determining excretion of xenobiotics.

Urinary Versus Biliary Excretion This point is best exemplified by the disposition of griseofulvin in rats and rabbits (Table 1.19). Rabbits excrete most of a dose of griseofulvin as 6-demethylgriseofulvin in urine. This is to be expected, because the molecular weight of this compound is only 328. According to Hirom *et al*. (1976), molecules with molecular weight (MW) < 350 tend to be preferentially excreted in urine, whereas those between 350 and 700 are predominately excreted in bile. Because the molecular

weight of griseofulvin conjugates is about 500, it is not surprising that rats, which biotransform griseofulvin extensively (phase II), excrete much of a dose in bile. This is an example of biotransformation being the critical step in the disposition of a xenobiotic. It is important to emphasize that alternative possibilities ought to be considered in any given instance to ensure that species differences are resolved in the disposition of xenobiotics. This point was illustrated by the work of Migdalof and colleagues, using captopril (Migdalof *et al*., 1984). This weak organic acid is predominately excreted in urine as the parent compound by both dogs and monkeys. It has negligible plasma protein binding and biliary excretion. Yet, urinary clearance of captopril is about three times as rapid in monkeys as in dogs. The authors resolved this species difference by determining that active tubular secretion of captopril is about three times higher in monkeys than in dogs.

Urinary Versus Fecal Excretion Often the elimination of a compound occurs by different routes in different species, as shown in the case of indomethacin in the dog and the rhesus monkey (Table 1.20). Dogs excrete most of a dose in feces, whereas monkeys excrete the majority of a dose in urine. Both species excrete similarly large quantities of a dose in bile. Because dogs excrete most of a dose in bile as conjugates (MW $>$ 500), it is to be expected that these hydrophilic indomethacin derivatives will not be reabsorbed unless they are hydrolyzed by intestinal bacteria to the reabsorbable parent compound or to phase I metabolites (which have good bioavailability). Based on available experimental data, it is not possible to decide with certainty whether or not this is occurring in the dog. It appears that indomethacin undergoes enterohepatic circulation with repeated conjugation in the liver and deconjugation in the small intestine, with a gradual "loss" of conjugates into the large intestine. However, because almost all of fecal excretion consists of indomethacin it is apparent that the large intestinal flora hydrolyzes the indomethacin conjugates. Limited reabsorption of indomethacin is not surprising (pK = 4.5, colon pH = 8), because more than 99.7% of indomethacin is ionized in the large intestine, which has a small surface area (compared to the small intestine). This does not allow for a sufficiently rapid shift in the mass balance to result in substantial reabsorption.

The monkey also reveals extensive enterohepatic recycling (57.7% of dose excreted in bile within 2 h). However, most of the biliary excretion consists of parent compound, which is readily reabsorbed. Furthermore, biliary conjugates appear to be hydrolyzed by small intestinal bacteria followed by reabsorption, because "loss" into feces is comparatively small (about 10% of dose). In contrast to the dog, monkeys excrete most of a dose as phase I metabolites (24.2% of dose) and indomethacin (10.5% of dose). Because indomethacin has a molecular weight of 358 and phase I metabolites have molecular weights of 220–345, these compounds are readily excreted in urine, as expected according to the work of Hirom and coworkers (Hirom *et al*., 1976).

TABLE 1.19 Urinary and Biliary Excretion of Griseofulvin and/or Metabolites in Rats and Rabbits[a]

	Rats[b]		Rabbits[b]	
	Urine	Bile	Urine	Bile
Total	12	77	78	11
Phase I metabolites	ND	23	70	3
Phase II metabolites	ND	54	8	8

[a]Modified from Symchowicz et al. (1967).
[b]Expressed as percentage of dose; ND, not determined.

1.5.7.3 Conclusions

An understanding of species differences in the disposition of xenobiotics is of utmost importance, because the time course of dispositional events in an organism can be the crucial factor in the manifestation of toxicities. Thus, interpretation and,

TABLE 1.20 Urinary, Biliary, and Fecal Excretion of Indomethacin and/or Its Metabolites in Dogs and Monkeys after IV Dosage[a]

Compound	Urine		Bile		Feces	
	Dog	Monkey	Dog	Monkey	Dog	Monkey
Indomethacin	0.6[b]	10.5	3.8	33.6	68.7	4
Phase I metabolites	4.1	24.2	NI	NI	2.7	6
Phase II metabolites	3.3	17.9	52.1	8.1	3.1	NI
Total dose excreted	7.9	52.7	55.9	51.7	76.3	10

[a]Modified from Hucker et al. (1966) and Yesair et al. (1970).
[b]Values represent percentage of dose excreted; NI, not identified or very small amounts.

more important, extrapolation of toxicity data from one species to another is only possible if the kinetics of a xenobiotic are known. These discussions demonstrate that any and each step in the disposition of xenobiotics may be of major or minor importance for a particular compound in a particular species. However, there is sufficient knowledge available that an informed investigator can resolve the kinetic cause of species differences for virtually any new chemical. More important, only a broad view of the disposition of xenobiotics, as detailed in this overview, will enable a vigilant investigator to avoid the pitfalls of personal bias toward one or the other step as more or less important in the disposition of chemicals.

Of utmost importance is knowledge of the half-life of pesticides for kinetic considerations in addition to the dose. Unfortunately, we do not have a compendium of critical data on pesticides as we do have it for drugs (Baselt, 2004).

1.5.8 Discussion of Factors Influencing Toxicity

The fact that a number of factors influence dosage-response relationships should not obscure the fact that these relationships are real and may be of importance.

No special study seems to have been made of the interrelation of factors in regard to ratios of difference. If the highest ratios observed for a series of factors were completely multiplicative in their effect, the combined product would be very large. Although the possibility of such an occurrence cannot be excluded, none has been recognized. On the average, the ratios expressed as quotients differ little from 1.0, and because some are less than 1.0, they tend to cancel out.

Table 1.21 summarizes part of the information in the foregoing sections. It is clear from this summary and from

TABLE 1.21 Summary of Information on the Importance of Different Factors Influencing Toxicity[a]

Factor	Total number of compounds	Ratio of difference		Increasing ratio indicates[b]
		Range	Mean	
Duration	22	0.5–20.0	—	2-year > 90-day
Route	67	0.2–21[c]	4.2[c]	Oral > dermal
Species	20	0.2–11.8	1[d]	Other species > rat
	1	>230		Other species > rat
	1	>1000		Human > cat
Individual[e]				
Oral route	69	1.20–7.14	2.42	LD 50 > LD 1
Dermal route	42	1.37–14.93	3.00	LD 50 > LD 1
Sex				
Oral route	65	0.21–4.62	0.94	Male > female
Dermal route	37	0.11–2.93	0.81	Male > female
Pregnancy	19	0.74–14.55	1.90	Pregnant > nonpregnant
Age	18	0.6–10.0	2.9	Newborn > adult
	16	0.7–6.2	—	Cold > warm
	15	0.2–4.1	1.8	Newborn > adult
	290	<0.02–750	2.78[f]	Newborn > adult
Temperature	1	10,000	—	Cold > warm
Nutrition	8	1.0–1.8	1.49	1/3 dietary protein > normal No protein > normal

[a]*Expanded from Hayes (1967a), by permission of the Royal Society, London.*
[b]*> indicates greater toxicity of chemical or greater susceptibility of animal.*
[c]*Compounds with very low or variable toxicity are not included.*
[d]*Approximate value.*
[e]*Same sex.*
[f]*Geometric mean.*

additional information in Tables 1.8 and 1.9 that species differences may be more important under practical conditions than any factor except dosage and time in influencing toxicity of a particular compound. The largest ratio of difference found in connection with species was over 1000 whereas the largest ratio associated with any other factor likely to be of practical importance was only 21. It is true that very large ratios have been observed in connection with age and temperature, respectively, but their rarity must be emphasized.

In summary, the maximal observed variation in effect associated with different factors is as follows: dosage and time-essentially infinite (health versus death) compounds-10^7, temperature-10^4, age-10^3, species-10^3, other factors-3×10 or less.

The numerical comparison regarding species ignores important phenomena that occur in humans but are difficult or impossible to study in animals. One is forced to conclude that more emphasis should be placed on studies in humans. This is particularly true when one considers that not only dosage and time and route (which includes duration) but sometimes sex, age, temperature, duration of dosing, and other factors may be explored directly in volunteers or workers.

Compound (agent) and species (subject) determine the qualitative aspect of toxicity, whereas dose and time define the quantitative relationships of the interaction between subjects and agents. Dose- and time-dependences are modified by a multitude of factors discussed in this chapter, any one of which may under some circumstances impact on the $c \times t$ relationship.

Relative potency is an intrinsic property of compounds often called a structure-activity relationship. Structure-activity relationships are mostly limited to closely related chemical structures because only compounds exerting the same effect (by the same mechanism of action) can be part of a structure-activity relationship (requirement for constancy of effect).

Relative susceptibility is an intrinsic property of species for which the proper term in analogy to structure-activity would be species-reactivity. Species-reactivity relationships are often limited to closely related species because a given chemical (constancy of structure) will display a species-reactivity relationship only as long as it is acting by the same mechanism. Coining these new terms instead of using the traditional notion of species differences was necessitated by a concept that has gone awry. The original purpose of studying species differences was to understand and know of the similarities between species because predictions can be based only on similarities but not on differences. These days species differences are being studied in a *l'ars pour l'ars* fashion which led many ignorant would-be toxicologists to claim that species-to-species predictions are impossible.

As suggested by Hayes (1991) using limited human data obtained from volunteers or occupationally exposed workers in conjunction with detailed animal studies conducted under ideal conditions (Sections 1.1.1–1.1.4) allows highly predictive safety-risk assessments when the principles of toxicology are applied according to the law(s) of toxicology.

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