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Perspectives in Pharmacology

The Role of Time as a Quantifiable Variable of Toxicity and the Experimental Conditions When Haber's $c \times t$ Product Can Be Observed: Implications for Therapeutics

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ABSTRACT

One hundred years ago, Warren established for the first time a quantitative link between dose and time while studying the toxicity of sodium chloride in *Daphnia magna* (Straus). During this century, many toxicologists in different contexts returned to this idea, which has become known as Haber's Rule of inhalation toxicology. Most attempts to explore this relationship ended in frustration because of the observed deviations from it, which were unfortunately called exceptions. Thus, toxicologists concentrated on the quantitative relationship between dose and effect under mostly isotemporal conditions, while time was assigned such arbitrary, semiquantitative designations as acute, subacute, subchronic, and chronic. Time itself as a quantifiable variable of toxicity was seldom studied and when it was examined, it was often not done under isodosic (steady-state) conditions. A recent analysis of time as a variable of toxicity indicated the existence of at least three independent time scales (toxicokinetic, toxicodynamic, exposure frequency/duration) in toxicological studies, which interact with dose and effect to yield the enormous complexity known to every toxicologist. Based on prototypical examples when toxicokinetic (dioxins, chloroacetic acid), toxicodynamic (nitrosamines, soman, sarin, tabun), exposure frequency (methylene chloride), or other experimental design-related conditions ($HgCl_2$, $CdCl_2$) represent the critical time scale, the general validity of the $c \times t = k$ concept will be discussed as a starting point for a theory of toxicology. As endpoints of toxicity, (delayed) acute toxicity, blood dyscrasias, and cancer will be used to illustrate the critical conditions needed to demonstrate the validity of this theory. The relevance of this theory to the pharmacologic action of chemicals and its implication for the therapeutic index are also discussed.

This year is the centennial of Warren's (1900) article on the toxicity of sodium chloride in *Daphnia magna* (Straus), linking, for the first time, dose and time in a quantitative relationship: $(c - c_o) \times t = k$. Problems with this simple formula were noted soon thereafter, and Ostwald and Dernoscheck (1910) suggested in analogy to an adsorption isotherm that $c^x \times t = k$ provides a better description of experimental data. Haber (1924) used the simplest form of the dose/time relationship $(c \times t = k)$ to estimate the toxicity of war gases, and Flury and coworkers examined this phenomenon further when studying the toxicity of solvents (e.g., Flury and Wirth, 1934). Entomologists were the ones who confirmed most frequently the simplest $c \times t = k$ relationship, but they also reported departures from it. Bliss (1940) dealt with the deviations mathematically and concluded that departures from $c \times t = k$ can be described either by $c^x \times t = k$ or $c \times t^x = k$. Druckrey et al. (1967) studied the carcinogenicity of a large number of nitrosamines and came to the conclusion that the latency to cancer can be best characterized by $c \times t^x = k$, where in some instances $x = 1$. The relationship kept reappearing in different experimental contexts (e.g., Gardner et al., 1977, 1979), but each time anomalies showed up and the claim for generalization was given up because of the occurrence of presumed exceptions.

Claiming exceptions to an often observed phenomenon is detrimental to the scientific approach since it puts an end to further inquiry. The more appropriate question is why does a particular experiment show departure from the frequently made observation of $c \times t = k$? This generalized question arose as a result of experience with studying the toxicity of

ABBREVIATIONS: HpCDD, 1,2,3,4,6,7,8-heptachlorodibenzo-*p*-dioxin; TCDD, 2,3,7,8-tetracholorodibenzo-*p*-dioxin; MCA, monochloroacetic acid; DENA, diethylnitrosamine; OP, organophosphates.

dioxins. Why was the remarkably consistent $c \times t = k$ of 1,2,3,4,6,7,8-heptachlorodibenzo-*p*-dioxin (HpCDD) for delayed acute toxicity (Rozman, 1999) not observed with 2,3,7,8-tetracholorodibenzo-*p*-dioxin (TCDD)? It was entirely implausible that TCDD would be an "exception" from something that applies to HpCDD considering the perfect structure/activity relationships in terms of all other aspects of their effects. Therefore, the challenge was to identify a critical step(s) that was responsible for the manifestation of $c \times t = k$ in the case of HpCDD and the lack thereof in the case of TCDD.

Examination of the $c \times t$ Concept

Rozman and Doull (2000) recently suggested a decision tree-type analysis to identify critical steps in the toxicity of chemicals. Use of this analysis revealed that the critical difference between HpCDD and TCDD resided in their differential kinetics. In other instances, the analysis revealed dynamic step(s) as the crucial one(s) in the manifestation of toxicity. In either instance, frequency/duration of exposure needed to be carefully considered as an additional independent time scale. In the following discussion, examples will be used to illustrate under what circumstances Haber's Product will be obtainable when either kinetics, dynamics, or exposure frequency represents the rate-determining step in the development of toxicity.

Kinetics

If the kinetic half-life of a compound (as determined from its plasma disappearance profile) is longer than the dynamic half-life of the effect (as determined from its effect recovery profile), it will dominate the overall process of toxicity under conditions of intermittent exposure.

Case 1: Very Long Kinetic Half-Life. HpCDD yielded $c \times t = k$ in terms of delayed acute toxicity (Rozman, 1999), whereas TCDD did not (Stahl et al., 1992). The half-life of TCDD in female Sprague-Dawley rats is about 20 days and that of HpCDD about 200 to 300 days (Viluksela et al., 1997, 1998). Rats died as a result of wasting for up to 70 days after treatment with a single dose (rate) of HpCDD, but no rat died after 30 days when treated with a single dose (rate) of TCDD. The reason for this difference is that 70 days represents less than ⅓ of one half-life for HpCDD, whereas 30 days amounts to about 1½ half-lives for TCDD. Consequently there is a minimum departure from steady state (at the most 16%) regarding the body burden of HpCDD during the observation period, but there is a major departure from it (about 62%) in terms of that of TCDD. As a result, the single dose (rate) experiment with HpCDD was being conducted under nearly ideal conditions (near kinetic steady state), allowing for little recovery to occur during 70 days after dosing, whereas in TCDD-treated animals, a sufficient amount of chemical has been removed from the organism for significant recovery to have occurred during 30 days after dosing. When TCDD was administered to female rats under isoeffective conditions as a loading dose followed by maintenance doses every 4 days the $c \times t = k$ relationship emerged with clarity also for this dioxin congener (Fig. 1). It should be noted that this study was conducted under isoeffective conditions (100% mortality), which represents the "ideal" condition to study the relationship between dose and time. Variability is larger in this experiment than in the experiment with HpCDD (Rozman, 1999). The reason for this is that different rats of the same dose group often received a different number of maintenance dose rates and with that different doses due to different times to death of individual animals. The lesson from these and other experiments (lower doses) was that the slightest departure from ideal conditions (kinetic steady state or monotonic departure from it) has a major impact on the $c \times t = k$ product, making it indiscernible in most toxicological experiments. It appears that there are no exceptions to the $c \times t = k$ relationship under ideal conditions and that most toxicological experiments do have uncontrolled (hidden) variables. For example, the kinetics of chemicals have virtually never been controlled in toxicological experiments unless nature did it in the form of medium to longer kinetic half-lives when

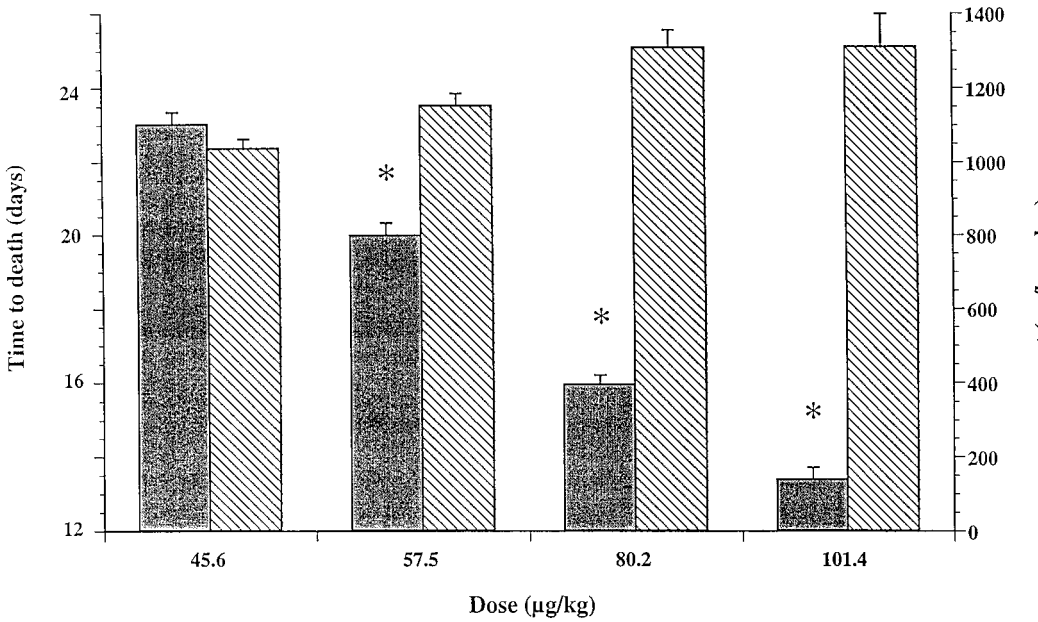

Fig. 1. Time to death and dose × time ($c \times t$) in TCDD-treated female Sprague-Dawley rats depicted on two separate scales as indicated by different ordinates. Time to death decreased significantly and incrementally with increasing dose (rates). The $c \times t$ product did not change much with increasing dose (rates). ■, time to death (days); ▨, $c \times t$ (µg/kg × day); *$p < 0.05$.

routine laboratory dosing regimens resulted in reasonably good steady-state concentrations.

Some might argue that the finding with HpCDD was just coincidental for another short-term effect and as such is just an exception from the exceptions regarding Haber's Rule. Rozman (2000b) showed the major chronic toxic effects of HpCDD in the continuation of the experiment that first addressed its delayed acute toxicity. The experimental design of that study has been described in detail in a previous publication (Rozman, 1999). Animals surviving the acutely toxic insult [including an acute no observable adverse effect level (NOAEL) of 2.5 mg/kg] developed anemia, lung cancer, liver cancer, various kidney pathologies, and a variety of low-incidence pathologies toward the end of the animal's natural life span, in the sequence as listed. Figure 2 demonstrates the chronic time response to various doses of HpCDD in terms of all causes of death. This chronic time response predicts with great accuracy the shortening of the animal's life span by increasing doses of HpCDD. It is truncated at about an LD_{80} by the delayed acute dose response that had killed most or all animals at higher doses. At least two of the effects were concurrently present in many animals (anemia and lung cancer), making separation of these two endpoints of toxicity difficult other than by the time to occurrence of effect in the first subject to be affected. However, if each effect occurs according to $c \times t = k$, then the sum of all effects must also be $c \times t = k$ for all subjects. This indeed has been confirmed for all chronic effects combined (Rozman, 2000b). The remarkably low variability is comparable to data obtained for delayed acute toxicity (Rozman, 1999). It should be noted that these chronic effects developed while the animals were eliminating a significant portion of their body burden, allowing for increasing recovery to occur. With a half-life of about 200 days, 75% of HpCDD was eliminated by day 400 and 87.5% by day 600, a period during which most chronic deaths occurred. To elucidate the role of recovery in the toxicity of HpCDD, the compound was also administered as a loading dose rate (2.8 mg/kg) followed by biweekly maintenance dose rates (0.085 mg/kg) to maintain kinetic steady state. Even though this dosing regimen increased considerably the dose of HpCDD, the $c \times t$ product remained approximately constant because of an entirely predictable shortening of the animal's life span (Rozman, 2000b). The second important message of this experiment is the increased incidence of anemia and lung cancer and the unchanged/decreased incidence of liver cancer and kidney pathologies with the complete disappearance of other pathologies (Rozman, 2000b). This important finding is consistent with the notion that some rats recovered sufficiently after the single dose rate to live considerably longer than under conditions of kinetic steady state. Because of this recovery, they did not develop anemia and lung cancer but contracted other toxicities/pathologies, which could not develop under conditions of kinetic steady state because of the shortening of the animal's life span.

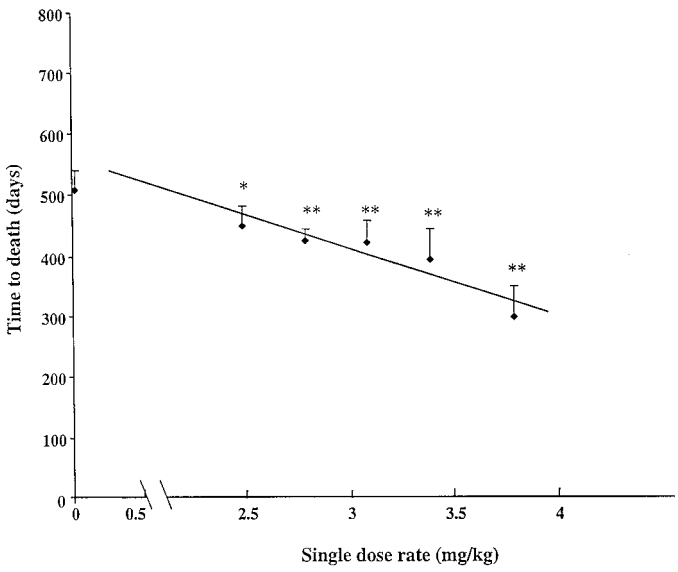

Fig. 2. A plot of time to death versus dose in female Sprague-Dawley rats chronically exposed to HpCDD by administration of a single dose (rate). Increasing dose (rates) significantly and incrementally decreased time to death as compared with controls. $^*p < 0.05$; $^{**}p < 0.01$.

Case 2: Intermediate Kinetic Half-Life. Monochloroacetic acid (MCA) has a half-life of about 2 h in male rats and a time to effect (coma, death) of the same order of magnitude. Therefore, in the course of the development of toxicity, a significant portion of a dose will be eliminated allowing for some recovery to occur while toxicity is developing. Like TCDD, MCA does not obey Haber's Rule when administered as a single dose (rate), although it comes close to it after subcutaneous injection (Hayes et al., 1973) because of the slow release of MCA from this depot. To create ideal conditions, MCA had to be infused by osmotic mini pumps because repeated administration of maintenance dose rates by most of the other commonly used methods would have severely disturbed the animals, triggering convulsions (and death) and thereby off-setting their normal time schedule to develop coma according to $c \times t$. Rozman (2000) demonstrated that this experimental adjustment resulted in a reasonably good $c \times t = k$ relationship also for this compound and for still another endpoint of toxicity (coma). It must be recognized that it is not the "absolute" time scale that determines whether or not $c \times t = k$ will become manifest when steady state is not carefully controlled but the ratio between the half-life of a compound and the observation period. If the observation period is much shorter than the half-life of a compound, then $c \times t = k$ will be observable unless, for example, adaptation introduces a "hidden" variable. However, the more unfavorable this ratio becomes, the more recovery will be occurring concurrently with the development of toxicity. This will not only introduce an uncontrolled variable but also flatten the dose-and-time responses.

Case 3: Very Short Kinetic Half-Life. Methylene chloride is an example for such compounds, having an estimated kinetic half-life of 5 to 40 min. The following considerations, however, also apply to compounds of even shorter half-lives like ozone, although for ozone the dynamics of recovery may be rate-determining. For compounds of very short kinetic or dynamic half-lives, the distinction between kinetics/dynamics becomes less important because rapid elimination/recovery reduces time dependence of toxicity. Such compounds will be more concentration-dependent after any type of discontinuous exposure regimen, and only continuous exposure till the actual occurrence of an effect will yield $c \times t = k$. This is probably the origin of the notion that Haber's Rule applies to inhalation toxicology only, when in fact inhalation happens to be the only practical way (intravenous infusion for days to

months is clearly not) to provide continuous exposure for compounds having very short kinetic or dynamic half-lives. Here, the important factor is not inhalation but the need for continuous exposure. Intermittent exposure (6 h per day) to kinetically acting compounds will yield $c \times t = k$ only if their kinetic half-lives are at least a day or longer to allow for a reasonable steady state with little kinetic recovery (elimination) occurring between exposure episodes. If intermittent exposure is further fragmented by weekends, the half-life has to be correspondingly longer.

It needs to be emphasized that $c \times t = k$ may not be observable for compounds of very short kinetic half-life, but not because they are exceptions from this law of toxicology as claimed by some scientists (Boyes et al., 2000). If exposure is not continuous until the actual measurement of an effect, then the departure from $c \times t = k$ will depend on the ratio between the half-life of a compound and the elapse of time between cessation of exposure and the actual measurement taken. Often the lack of a good time resolution will make it difficult to design experiments to demonstrate $c \times t = k$. However, when time resolution is good, as in the pungency of acids, clear summation effect can be observed on a time scale of seconds (Cometto-Muñiz and Cain, 1984).

Dynamics

For compounds causing effects such that the effect recovery half-life is longer (slower recovery) than the kinetic half-life, the former will dominate the dynamics of the effect. These are the hit-and-run type poisons, which can be eliminated very rapidly and yet produce an effect in accordance with $c \times t = k$.

Case 1: Very Long Dynamic Half-Life. There are few examples of recovery taking place on a time scale of years or longer. Chemical neuropathies are the closest examples that come to mind. Because of the enormous reserve capacity and plasticity of the nervous system, it is difficult to conduct conclusive studies in this area. As is the case for compounds with very long kinetic half-lives, both the frequency/duration and the recovery can be critical for these compounds, depending on the dynamics of the effect. If the damage is highly irreversible as occurs with Ginger Jake paralysis (Morgan and Penovich, 1978), or with methanol's damage to the retina/optic nerve, accumulation of injury will occur according to a triangular geometry after repeated above-threshold exposures in spite of the short kinetic half-lives of these compounds. If an essentially irreversible injury is very severe, it is very difficult to titrate the dose/injury to assure reproducible survival, which is necessary when studying time to effect phenomena.

Case 2: Intermediate Dynamic Half-Life. Diethylnitrosamine (DENA) has a short kinetic half-life of about 10 min in rats (Druckrey et al., 1967). Feeding rats a diet with different daily dose rates resulted in a reasonably good $c \times t = k$ relationship with cancer as endpoint of toxicity (Druckrey et al., 1963; Rozman, 2000b). The daily dose rate was a poor surrogate of dose (cumulative) because of the vastly different life span of the animals receiving the various daily dose rates. It is worthwhile to note that $c \times t = k$ is less variable under isoeffective conditions than when departure from it takes place (see 91 and 64 mg/kg doses). Again, with a kinetic half-life of 10 min for DENA and two bouts of feeding per day, no good $c \times t = k$ relationship should be expected if kinetics were rate-determining because of very rapid kinetic recovery (elimination) after completion of absorption of each daily dose rate. However, in general, the half-life of DNA adducts of potent carcinogens is in the order of weeks to months (Szafarz and Weisburger, 1969; Swenberg et al., 1985; Pitot and Dragan, 1996), which provides the key to understanding the reasonably good $c \times t = k$ obtained (Rozman, 2000b). The dynamics of DENA-induced cancer are dominated by the long dynamic half-life of the effect and not by the short kinetic half-life of the causative agent. Thus, animals exposed to DENA in the diet are not at kinetic steady state at all, but they are at dynamic steady state with regard to the DNA damage, which is the reason for the good $c \times t = k$ relationship.

Case 3: Short Dynamic Half-Life. The kinetic half-life of soman, sarin, and tabun is about 10 min to 1 h in all species studied, but the recovery half-life from intoxication is about 12 h (Lintern et al., 1998; Rozman, 2000b). Considering the ratio between observation period (6 h) and kinetic half-life (10 to 60 min) suggests extremely unfavorable conditions for $c \times t = k$ to occur due to virtually complete kinetic recovery by the end of the observation period. In fact, there is an excellent $c \times t = k$ relationship (Sivam et al. 1984), at least for those organophosphates (OP) that display slow recovery, because it is the dynamic half-life of the effect and not the kinetic half-life of the compound that determines $c \times t = k$.

There are two important issues here that indicate the relevance of time as a variable also of therapeutics. A case in point is antidotal therapy in OP poisonings. In terms of dose alone, soman is the most toxic OP among the three OPs listed above; however, in terms of dose and time, both tabun and sarin are more dangerous than soman because the time to death is shorter for sarin and much shorter for tabun than for soman (Rozman, 2000b). Indeed, a severely intoxicated person's life was saved by administering atropin sulfate and PAM (pralidoxime, 2-formyl-*N*-methyl-pyridinium chloride oxime) within 5 to 10 min of an accident with soman (Sidell, 1974). It is unlikely that this person's life could have been saved if the agent in this intoxication episode had been tabun because of the short therapeutic window (15 min) in terms of time.

Many if not most therapeutic agents belong to the last two cases discussed (short to intermediate dynamic half-life). Consider the H^+-ion pump inhibitor omeprazole. The binding half-life to its receptor is 24 h. Its kinetic half-life is about 1 h or less. If the therapeutic dose would be based on its kinetic half-live, the conclusion would be that after 6.64 half-lives (6.64 h) 99% of the drug would be eliminated. Therefore, a dosing regimen entailing the administration of the drug every 6 h still would yield a very poor steady state and, with that, moderate to low therapeutic efficacy. Omeprazole, in fact, is given daily once or every other day in accordance with its pharmacodynamic half-life of 24 h. It has been reported that it takes 3 days for maximum effect (clinically indistinguishable from 3.32 dynamic half-lives equaling 90% of steady state) and 3 to 5 days for cessation of effect, which is clinically also indistinguishable from 3.32 reversibility half-lives. The action of this drug is entirely dominated by dynamics, and any opinion based on kinetics would be in error.

Special Cases: Experiment-Driven Steady State. Any time an experiment is conducted under conditions of kinetic

steady state (e.g., continuous inhalation until observation beyond four to seven kinetic and/or dynamic half-lives) the outcome will be in accordance with Haber's Rule unless some hidden variables are impeding the outcome. In vitro studies, experiments involving the use of aquatic species (fish, pond snails, etc.), or any instance when continuous exposure is implicitly part of the experimental design will result in experiments showing simple or more complex $c \times t = k$ relationships unless kinetic or dynamic adaptation(s) occur during measurement of toxicity (Verhaar et al., 1999). In fact, Rozman (2000b) provided an indication for the accuracy of the $c \times t = k$ relationship using sea urchin sperm motility as endpoint of toxicity and divalent cations as toxicants.

Conclusions

If physicists would have claimed the occurrence of exceptions to what later became Boyle's Law of ideal gases as deviations from it started accumulating after some initial confirmatory evidence, mankind would not possess perhaps the most profound theory ever devised, which is thermodynamics. The greatest impediment in the development of the discipline of toxicology was to view deviations from the $c \times t = k$ concept as exceptions. Claiming that an exception exists stifles any attempt at generalization, which eventually is needed to develop a theory, which in turn is a precondition of a scientific discipline. If the discipline of toxicology is to develop into a science, then the prerequisite is to change this notion about exceptions. There are no exceptions to a fundamental law of nature, and if we assumed that $c \times t = k$ under isoeffective conditions or $c \times t = k \times E$ (effect) under isodosic or isotemporal conditions represents a law of toxicology, then there should be no exceptions to it either (for detail, see Rozman, 2000a and Rozman and Doull, 2000). What we then need to understand are the conditions under which $c \times t = k$ or $c \times t = k \times E$ can be experimentally observed to begin to explore apparent departures from it by asking the following questions.

Why does it appear that this or that experiment deviates from the $c \times t$ concept? What are the uncontrolled (hidden) variables? What experiments are needed to find out?

Short of changing our experiments to accommodate these questions, there will be little real progress in toxicology as a science. Toxicology was first marginalized by risk assessment because of the claim that it could not answer conclusively questions about high to low dose extrapolation and species-to-species interpretation. Unfortunately, toxicologists did not challenge forcefully enough this notion on grounds of the $c \times t$ concept, which does allow to define both the dose and the time threshold within biological variability (normal distribution). This led risk assessors to the absurd assumption (from the biological point of view) that time is not important at low doses (Crump et al., 1976) when in fact time is less important at high doses but it dominates the low dose end of the $c \times t$ relationship. The recently emerging precautionary principle (to lower exposure as much as possible) will complete this process by making the large scale toxicological testing/evaluations of environmental chemicals a matter of the past. Perhaps this is the only way to confront currently accepted experimental designs (acute, subchronic, chronic toxicity), which ignore time as a quantifiable variable of toxicity. The analysis provided in this article suggests that it is possible to understand the conditions under which Haber's Rule can be experimentally demonstrated that would make this relationship into a law of toxicology. Toxicology has benefited from pharmacology immensely both methodologically and theoretically. As continuous long-term therapies become more and more common, perhaps pharmacology can take advantage of these insights gained in toxicological investigations and examine its implications for beneficial rather than adverse effects, both of which are clearly subject to the same fundamental laws of nature.

One such pharmacological principle that could benefit from application of Haber's Rule is the therapeutic index. The therapeutic index is traditionally defined as the ratio of the toxic dose to the therapeutic dose (LD_{50}/ED_{50}). Dose in this expression could be viewed as a surrogate for exposure, but generally time is ignored although it is an equally important variable of exposure. Dose is a simple variable (number of molecules reaching the receptor), whereas time is a complex variable with kinetic, dynamic, and frequency/duration scales.

If we establish the minimal combinations of dose and time that are required to produce a specific toxic or therapeutic effect with continuous exposure, we can plot the logs of these time and dose values to obtain a slope of -1 (Fig. 3). Parallel lines will be obtained for all other effects of an agent, and all of these lines can be described by Haber's Rule ($c \times t = k$) under ideal exposure conditions. Each of these lines defines the individual or population exposure threshold for a specific therapeutic or toxic effect, and the ends of these lines are defined by those combinations where further increases of dose no longer reduce time to effect or conversely where further increases in time no longer reduce the dose required to produce the specific effect. The k values for specific toxic and therapeutic effects can be used as surrogates for exposure to define the therapeutic index

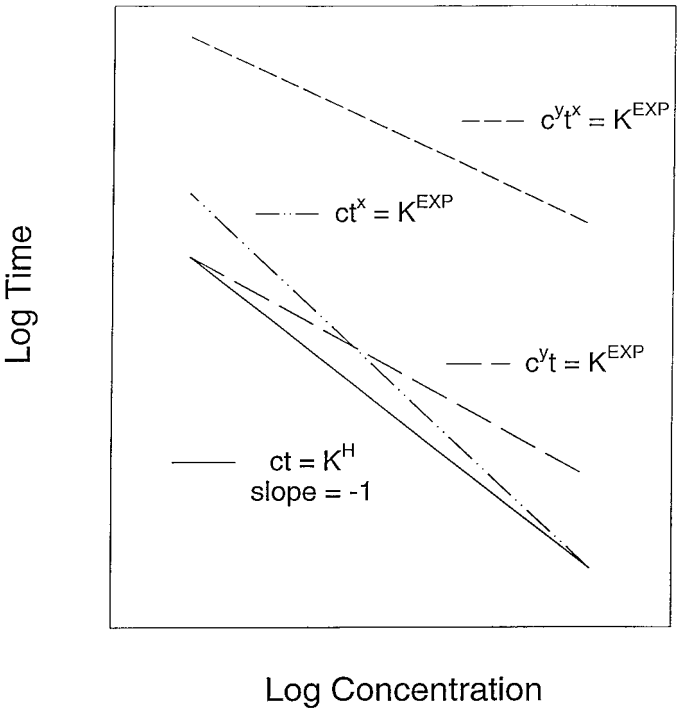

Fig. 3. Relationship between dose and time under different conditions of exposure.

of an agent in the same way as is done currently with dose (therapeutic index = $k^{\text{toxic effect}}/k^{\text{therapeutic effect}}$). When the effects are the result of long-lasting or slowly reversible dynamics or kinetics, continuous exposure is not needed to establish Haber's k values. Fractionation of the time or dose will result in lines with different slopes and k values ($c \times t^y = k^t$, $c^y \times t = k^c$) as will fractionation of both dose and time ($c^x \times t^y = k^{ct}$) (Fig. 3), but these k values could also be used to characterize the therapeutic index by comparing conditions of intended usage versus minimum toxicity under conditions of continuous exposure.

Many more examples of the potential importance of time as a quantifiable variable of therapeutics could be provided, but the intent of this article is not comprehensiveness but the stimulation of discussion with pharmacologists regarding the applicability of Haber's Product to both disciplines.

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