chapter two

Why is a toxicant poisonous?

Theophrastus Bombastus von Hohenheim, better known in history as Paracelsus, who was born in the Swiss village of Einsiedeln in 1493 and died in 1541, taught us that the severity of a poison was related to the dose (see Strathern, 2000). His citation "All substances are poisons; there is none which is not a poison. The right dose differentiates a poison from a remedy" is found in the first chapter of almost all textbooks of toxicology or pharmacology. However, the molecular theory was formulated more than 300 years later, and the law of mass action not until after the middle of the 19th century. Real rational toxicology and pharmacology are dependent on these laws, and hence could not develop properly before they were known.

Paracelsus' idea that all substances are poisons is, of course, correct; even water, air, and sugar are poisons in sufficient amounts, but by looking at the chemical structures of typical poisons, and trying to sort out the reactions they tend to be involved in, we can roughly put them into seven categories. By using the molecular theory, the law of mass action, and our knowledge of the nature of the chemical processes in organisms, we can condense biochemical toxicology to three sentences, and about seven types of reactions:

1. Toxic molecules react with biomolecules according to the common laws of chemistry and physics, so that normal processes are disturbed.
2. The symptoms increase in severity with increasing concentration of the toxicant at the site of reaction.
3. This concentration increases with increasing dose.

2.1 Seven routes to death

The chemist may prefer to classify toxicants according to their chemical structure, the doctor according to the organ they harm, the environmentalist according to their stability in the environment, and so forth. The biochemist may use a different classification, and we will approach the toxicology of pesticides from the biochemist's perspective. Because of point 1 above, and because the cells in all organisms are very similar, it is possible to classify

toxicants into roughly seven categories according to the type of biomolecule they react with. Toxicants in the same category do not need to be chemically related, and one substance may act through several mechanisms. The following simple classification is based on the more comprehensive texts of Ecobichon (2001) and Gregus and Klaassen (2001).

2.1.1 Enzyme inhibitors

The toxicant may react with an enzyme or a transport protein and inhibit its normal function. Enzymes may be inhibited by a compound that has a similar, but not identical structure as the true substrate; instead of being processed, it blocks the enzyme. Typical toxicants of this kind are the carbamates and the organophosphorus insecticides that inhibit the enzyme acetyl cholinesterase. Some extremely efficient herbicides that inhibit enzymes important for amino acid synthesis in plants, e.g., glyphosate and glufosinate, are other good examples in this category.

Enzyme inhibitors may or may not be very selective, and their effects depend on the importance of the enzyme in different organisms. Plants lack a nervous system and acetylcholinesterase does not play an important role in other processes, whereas essential amino acids are not produced in animals. Glyphosate and other inhibitors of amino acid synthesis are therefore much less toxic in animals than in plants, and the opposite is true for the organophosphorus and carbamate insecticides.

Sulfhydryl groups are often found in the active site of enzymes. Substances such as the Hg^{++} ion have a very strong affinity to sulfur and will therefore inhibit most enzymes with such groups, although the mercury ion does not resemble the substrate. In this case, the selectivity is low.

2.1.2 Disturbance of the chemical signal systems

Organisms use chemicals to transmit messages at all levels of organization, and there are a variety of substances that interfere with the normal functioning of these systems. Toxicants, which disturb signal systems, are very often extremely potent, and often more selective than the other categories of poisons. These toxicants may act by imitating the true signal substances, and thus transmit a signal too strongly, too long lasting, or at a wrong time. Such poisons are called *agonists*. A typical agonist is nicotine, which gives signals similar to acetylcholine in the nervous system, but is not eliminated by acetylcholinesterase after having given the signal. Other quite different agonists are the herbicide 2,4-D and other aryloxyalkanoic acids that mimic the plant hormone auxin. They are used as herbicides. An *antagonist* blocks the receptor site for the true signal substance. A typical antagonist is succinylcholin, which blocks the contact between the nerve and the muscle fibers by reacting with the acetylcholine receptor, preventing acetylcholine from transmitting the signal. Some agonists act at intracellular signal systems. One of the strongest man-made toxicants, 2,3,7,8-tetrachlorodibenzodioxin, or dioxin, is a good

example. It activates the so-called *Ah* receptor in vertebrates, inducing several enzymes such as CYP1A1 (see p. 181). Organisms use a complicated chemical system for communication between individuals of the same species. These substances are called *pheromones*. Good examples are the complicated system of chemicals produced by bark beetles in order to attract other individuals to the same tree so that they can kill them and make them suitable as substrates. Man-made analogues of these pheromones placed in traps are examples of poisons of this category. The *kairomons* are chemical signals released by individuals of one species in order to attract or deter individuals of another. The plants' scents released to attract pollinators are good examples.

Signals given unintentionally by prey or a parasite host, which attract the praying or parasitizing animal, are important. A good example is CO_2 released by humans, which attracts mosquitoes. The mosquito repellent blocks the receptors in the scent organ of mosquitoes.

2.1.3 *Toxicants that generate very reactive molecules that destroy cellular components*

Most redox reactions involve exchange of two electrons. However, quite a few substances can be oxidized or reduced by one-electron transfer, and reactive intermediates can be formed. Oxygen is very often involved in such reactions. The classical example of a free radical-producing poison is the herbicide *paraquat*, which steals an electron from the electron transport chain in mitochondria or chloroplasts and delivers it to molecular oxygen. The superoxide anion produced may react with hydrogen superoxide in a reaction called the Fenton reaction, producing hydroxyl radicals. This radical is extremely aggressive, attacking the first molecule it meets, no matter what it is. A chain reaction is started and many biomolecules can be destroyed by just one hydroxyl radical. Because one paraquat molecule can produce many superoxide anions, it is not difficult to understand that this substance is toxic. *Copper* acts in a similar way because the cupric ion (Cu^{++}) can take up one electron to make the cuprous cation (Cu^{+}) and give this electron to oxygen, producing the superoxide anion ($O_2\cdot^{-}$).

Free radical producers are seldom selective poisons. They work as an avalanche that destroys membranes, nucleic acids, and other cell structures. Fortunately, the organisms have a strong defense system developed during some billion years of aerobic life.

2.1.4 *Weak organic bases or acids that degrade the pH gradients across membranes*

Substances may be toxic because they dissolve in the mitochondrial membrane of the cell and are able to pick up an H^{+} ion at the more acid outside, before delivering it at the more alkaline inside. The pH difference is very important for the energy production in mitochondria and chloroplasts, and

this can be seriously disturbed. Substances like *ammonia*, *phenols*, and *acetic acid* owe their toxicity to this mechanism. Selectivity is obtained through different protective mechanisms. In plants, ammonia is detoxified by glutamine formation, whereas mammals make urea in the ornithine cycle. Acetic acid is metabolized through the citric acid cycle, whereas phenols can be conjugated to sulfate or glucuronic acid. Phenols are usually very toxic to invertebrates, and many plants use phenols as defense substances.

2.1.5 Toxicants that dissolve in lipophilic membranes and disturb their physical structure

Lipophilic substances with low reactivity may dissolve in the cell membranes and change their physical characteristics. *Alcohols*, *petrol*, *aromatics*, *chlorinated hydrocarbons*, and many other substances show this kind of toxicity. Other, quite unrelated organic solvents like *toluene* give very similar toxic effects. Lipophilic substances may have additional mechanisms for their toxicity. Examples are *hexane*, which is metabolized to 2,5-*hexandion*, a nerve poison, and *methanol*, which is very toxic to primates.

2.1.6 Toxicants that disturb the electrolytic or osmotic balance or the pH

Sodium chloride and other salts are essential but may upset the ionic balance and osmotic pressure if consumed in too high doses. Babies, small birds, and small mammals are very sensitive. Too much or too little in the water will kill aquatic organisms.

2.1.7 Strong electrophiles, alkalis, acids, oxidants, or reductants that destroy tissue, DNA, or proteins

Caustic substances like strong acids, strong alkalis, bromine, chlorine gas, etc., are toxic because they dissolve and destroy tissue. Many accidents happen because of carelessness with such substances, but in ecotoxicology they are perhaps not so important. More interest is focused on *electrophilic* substances that may react with DNA and induce cancer. Such substances are very often formed by transformation of harmless substances within the body. Their production, occurrence, and protection mechanisms will be described in some detail later.

2.2 How to measure toxicity

2.2.1 Endpoints

In order to measure toxicity, it is important to know what to look for. We must have an *endpoint* for the test. An endpoint can be very precise and easy

to monitor, such as death, or more sophisticated, for instance, lower learning ability or higher risk for contracting a disease. Some endpoints are all-or-none endpoints. At a particular dose some individuals will then get the symptoms specified in the definition of the endpoint and others do not. Tumors or death are such all-or-none endpoints. Such endpoints are often called *stochastic*, whereas endpoints that all individuals reach, to varying but dose-dependent degrees, are called deterministic endpoints. Intoxication by alcohol is a good example. We use the term *response* for the stochastic all-or-none endpoints and the term *effect* for gradual endpoints.

2.2.1.1 Endpoints in ecotoxicology and pest control

The fundamental endpoints for nonhuman organisms are:

- Death
- Reduced reproduction
- Reduced growth
- Behavioral change

These endpoints are, of course, connected.

Reduced reproduction is probably the most important endpoint in ecotoxicological risk assessments, whereas in pest control, death or changes in behavior are the most important. We simply want to kill the pest or make it run away. Toxicity tests are often based on what we call surrogate endpoints. We measure the level of an enzyme and how its activity is increased (e.g., CYP1A1) or reduced (acetylcholinesterase), how a toxicant reduces the light of a phosphorescent bacterium, or how much a bacterium mutates. Such endpoints are not always intuitively relevant to human health or environmental quality, but much research is done in order to find easy and relevant endpoints other than the fundamental ones.

2.2.1.2 Endpoints in human toxicology

In human toxicology, we have a lot more sophisticated endpoints related to our well-being and health. At the moment, cancer is the most feared effect of chemicals, and tests that can reveal a chemical's carcinogenicity are always carried out for new pesticides. Other tests that may reveal possible effects on reproduction and on the fetus are important. Endpoints such as immunodeficiency, reduced intelligence, or other detrimental neurological effects will play an important role in the future. The problem is that almost all endpoints in human toxicology are surrogate endpoints, and elaborate and dubious extrapolations must be done. The new techniques under development that make it possible to determine the expression of thousands of genes by a simple test will very soon be used in toxicological research, but the interpretation problems will be formidable.

2.2.2 Dose and effect

The law of mass action tells us that the amount of reaction products and the velocity of a chemical reaction increase with the concentrations of the reactants. This means that there is always a positive relation between dose and the degree of poisoning. A greater dose gives a greater concentration of the toxicant around the biomolecules and therefore more serious symptoms because more biomolecules react with the toxicant and at a higher speed. This simple and fundamental law of mass action is one of the reasons why a chemist does not believe in homeopathy. It is also the reason why Paracelsus (1493–1541) was right when saying "All substances are poisons; there is none which is not a poison. The right dose differentiates a poison from a remedy" (Strathern, 2000). The connection between dose or concentration of the toxicant and the severity of the symptoms is fundamental in toxicology. By using the law of mass action, we get the following equilibrium and mathematical expression:

$$B + T \underset{}{\overset{K}{\rightleftharpoons}} BT$$

$$K = \frac{C_B \cdot C_T}{C_{BT}} \text{ or } C_B = K \cdot \frac{C}{C_T + K} \text{ if } C = C_B + C_{BT}$$

The target biomolecule (B) at the concentration C_B reacts with the toxicant (T) at the concentration C_T to give the destroyed biomolecule (BT) at the concentration C_{BT}. The reaction may be reversible, as indicated by the double arrow. C is the total concentration of the biomolecule and K is the equilibrium constant. If the onset speed of the symptoms is proportional with the disappearance rate of the biomolecules ($-dC_B/dt$), we get this simple mathematical expression telling us that the higher the concentration of the toxicant is, the faster C_B will decrease and the symptoms appear:

$$-\frac{dC_B}{dt} = k_{+1} \cdot C_B \cdot C_T$$

k_{+1} is the velocity constant for the reaction.

These simple formulae illustrate that higher concentrations of a toxicant give a lower amount of the biomolecule and thus stronger symptoms. The onset of symptoms may start when C_B is under a certain threshold or C_{BT} is above a threshold.

The real situation is more complicated. The toxicant may react with many different types of biomolecules. It may be detoxified or need to be transformed to other molecules before reacting with the target biomolecule.

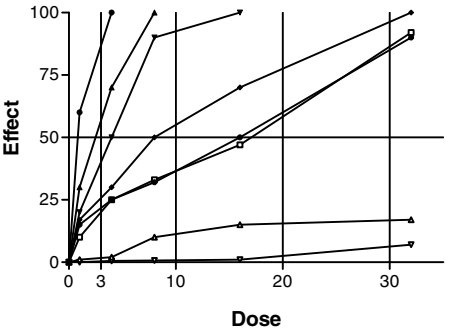


Figure 2.1 A hypothetical example of the effects on eight individuals of a toxicant at different doses.

2.2.3 Dose and response

The sensitivity of the individuals in a group is different due to genetic heterogenicity as well as difference in sex, age, earlier exposure, etc. Therefore, if the effect of a toxicant is plotted against the dose, every individual will get a curve that is more or less different from those of other individuals. In Figure 2.1, some effect on eight individuals is shown. The difference is exaggerated in order to elucidate the points.

Figure 2.1 illustrates a hypothetical example. The effect may be any measurable symptom that has a graded severity. Three individuals seem to be very sensitive, whereas one or two are almost resistant. This figure leads us to a very important concept called *response*. Response (r) is defined as the number of individuals getting symptoms higher than a defined threshold. If we decide that the symptom threshold should be 50, we observe that at doses 3, 10, 20, and 30 the response will be 2, 4, 6, and 6, respectively. When determining the response, we just count how many individuals have the required or higher symptoms.

The relative response (p) is the number of responding individuals divided by the total number given a certain dose. At the marked dose levels in Figure 2.1, the relative responses are 0.25, 0.5, 0.75, and 0.75, respectively. These numbers may be multiplied by 100 to give the percent response.

We very often measure all-or-none symptoms (dead or alive, with tumor or without tumor, numbers of fetus with injury or normal ones) in toxicology. Such symptoms are not gradual. We then have to expose groups of individuals with different doses (D) and determine the number of responding individuals (r) and the relative number (p).

If we have many groups with a high number of individuals and then plot the relative response against the dose, we very often get an oblique

S-shaped graph, with an inflection point at 50% response. The graph may be made symmetrical by plotting *log dose* instead of dose. Furthermore, the S-shaped graphs can be changed into straight lines by transforming the responses to *probit response*. We then presuppose that the sensitivity of the organisms has a normal distribution, which predicts that most individuals have average sensitivity, a few are very robust, very few are almost resistant, and some have high sensitivity.

The log transformation of dose or concentration is easily done with a pocket calculator. Using the formula for the inverse normal distribution in the data sheet *Excel*, one can easily do the calculation of the probit values. The mean or median is set to 5 and the standard deviation to 1, i.e., the formula will look like this:

=NORMINV (relative response; 5 ;1)

By writing the relative response into the formula, Excel will return the probit value.

Note that the probit of 0.5 (50% response) is 5, and the probit of 0.9 (90% response) is 6.282. The reader should try other values if Excel is available. Note also that the probit of 0 is $-\infty$, whereas the probit of 1 is $+\infty$. Values of 0 or 100% response are therefore useless in this plot. Figure 2.2 and Figure 2.3 show the essence of some dose–response curves.

Figure 2.3a and b shows a case with sensitive and resistant flies mixed 50:50. The same data are used in both plots.

Figure 2.2a to c and Figure 2.3a and b show that the transformation of the doses to log dose, and the use of probit units for responses, makes it much easier to interpret the graphs. However, there are several difficulties with dose–response graphs.

Mathematically, the probit of a value P is Y in the integral

$$P = \frac{1}{\sqrt{2\pi}} \int_{-\infty}^{Y-5} e^{-\frac{1}{2}u^2} du$$

It cannot be expressed as a simple function, and some mathematical skill is necessary to interpret its meaning. Therefore, the much simpler logit transformation $L = \ln\{P/(1 - P)\}$ is often used. The logit values (L) can be calculated from the relative response values (P) with a pocket calculator. The logit transformation also gives almost straight lines if the sensitivity is normally distributed. The most serious problem with dose–response graphs, however, is not this mathematical inconvenience. The low reproducibility is a more serious problem. As an example, if you know exactly the LD50 (lethal dose in 50% of the population) and give this to 10 animals, the probability that 5 die is only 0.246. The confidence intervals of the responses for the same dose, or for the doses calculated to give a specified response (e.g.,

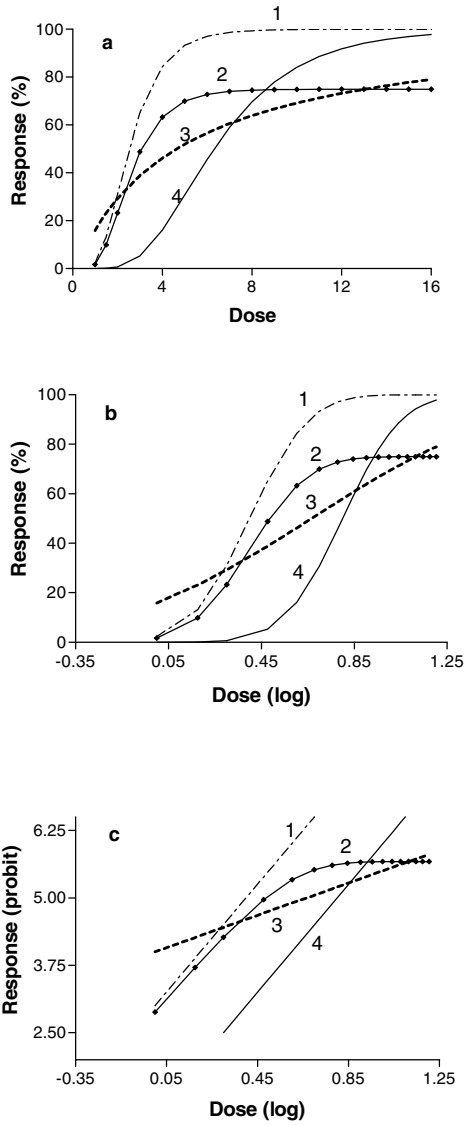


Figure 2.2 Dose–response relationships drawn on three different models for four populations. (a) Doses and responses in linear scale. (b) Doses in log scale and responses in linear scale. (c) Doses in log scale and responses in probits. (1) Sensitive population with normally distributed sensitivity and LD50 = 2.5 units. (2) A mixed population with 75% of (1) and 25% resistant individuals. (3) Intermediate sensitive population with normally distributed sensitivity, but more scattered than (1), and LD50 = 5 units. (4) Less sensitive, but normally distributed population, similar to (1), but with LD50 = 6.5 units.

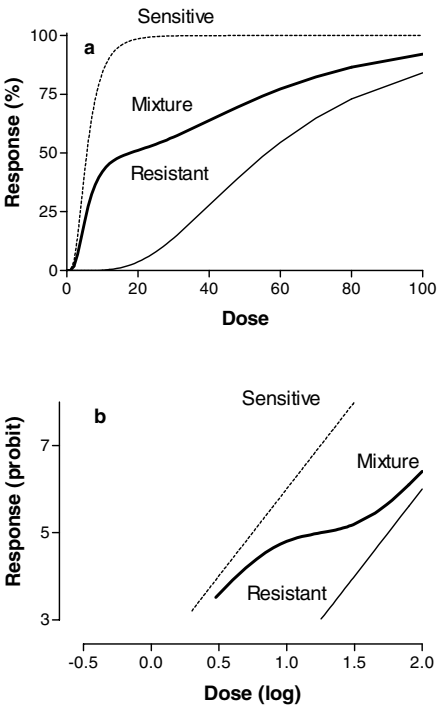


Figure 2.3 Dose–response curves for susceptible and resistant flies and a mixture (50:50) of susceptible and resistant flies. (a) Doses and responses on linear scales. (b) Doses on log scale and responses on probit scale.

LD50), will be large and are not easily calculated without special data programs. Another problem is that responses of 0 or 100%, which very often occur in practical experiments, give probit (or logit) values of $-\infty$ or $+\infty$ that cannot be plotted into the diagram. The outcome of such an experiment may be disappointing if nice curves are expected. Let us look at a case study before describing the scatter problem in more detail. A standard description of probit analyses can be found in Finney (1971).

2.2.3.1 Dose–response curves for the stable fly

As a real-life example, we can use an experiment done by myself as part of my master's thesis in 1962 (Stenersen and Sømme, 1963). The stable fly (*Stomoxys calcitrans*) is an important insect pest in husbandry. In the Nordic countries it is an indoor pest, present as many small, partially isolated populations. From 1950 to 1965 it was controlled with DDT, but resistance soon became a problem. A strain (R) of stable fly resistant to the DDT and related insecticides such as DDD and methoxychlor was compared with a sensitive (S) strain. Males from the R strain were then crossed with females from the S strain and the offspring (F1 of S × R) were tested. They were as sensitive as the S strain. The F1 flies were allowed to interbreed and the

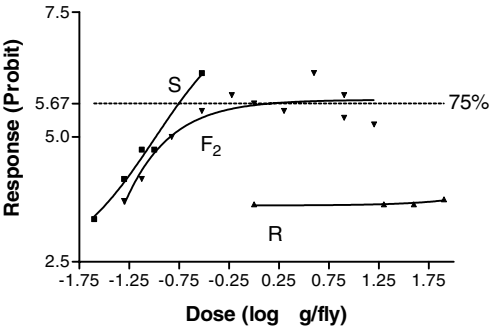


Figure 2.4 Dose–response relationships of *Stomoxys calcitrans* treated with the DDT analogue DDD. S, susceptible strain; R, resistant strain; F_2, second generation from crosses of S and R.

resulting F2 generation was tested. As seen from Figure 2.4, these flies had a very heterogeneous sensitivity against DDD. About 75% (probit value of 5.674490) were quite sensitive, whereas 25% were almost impossible to kill with DDD. This result is expected if just one (recessive) gene is involved in the resistance mechanism. The other DDT group insecticides gave similar results.

2.2.3.2 Scatter in dose–response data

The figure of the *Stomoxys* strains also illustrates the wide scatter expected for the response data. Each point is based on 20 individuals, i.e., more than 400 flies plus controls (60) were used in this small experiment. The scatter is formidable in spite of the great number of flies used. The reason is the stochastic nature of the outcome. For instance, the probability (P) of getting exactly 15 dead flies by using a dose that should kill 75% is only P = 0.203. It is much more probable that we get another "wrong" value. These results may be calculated from the binomial formula

$$P = \frac{n!}{(n-r)!\times r!} \times p^r \times (1-p)^{(n-r)}$$

where n = 20, number of insects tested in a group; r = 15, number of insects dying; p = 75/100, the expected value of relative response when a huge number of insects was used; and ! is the faculty sign (e.g., n! = n × (n – 1) × (n – 2) … 3 × 2 × 1). It may be calculated that P = 0.203, which is the probability of obtaining a response of r = 15 in an experiment where p = 0.75 and n = 20. An outlier (see Figure 2.4), as that obtained at 4 µg/fly (log dose = 0.602), with a response of r = 18 (90% mortality instead of the expected 75%), has a probability of 0.0669, i.e., is expected in as many as 7 experiments out of 100. Such uncertainties are inherent in dose–response relationships and have nothing to do with experimental errors, which may also be a source of scattering.

2.2.4 LD50 and related parameters

The statistical problems in making good dose–response curves can only be overcome by using many organisms in the experiment. A better method may be to determine one dose, for instance, the dose that is expected to kill or harm 50% of the individuals, and not to construct a graph. This can be done satisfactorily with much fewer individuals. The latter method is definitely better when studying vertebrates. Most countries have strict legislation concerning the use of vertebrates in research, and it is difficult to get permission to do experiments involving hundreds of animals. Furthermore, most vertebrates suitable for research are expensive. Therefore, we seldom find graphs of dose–response relationships on mammals in the more recent scientific literature. More often, we find a value called LD50 that can be determined with reasonable accuracy by using few individuals. LD50 is the dose expected to kill half of the exposed individuals. Sometimes we are interested in determining the doses that kill 90 or 10%, etc., and these doses are called LD90 and LD10, respectively. They can easily be determined from a dose–response curve, but these values are less accurate than LD50. If we study endpoints other than death, we use the term ED50 (effective dose in 50% of the population), and if we study *concentrations* and not doses, we use the terms LC50 (lethal concentration in 50% of the population) and EC50 (effective concentration in 50% of the population). Protocols for determination of LD50 for rodents are available in order to minimize the number of animals necessary for a satisfactory determination. According to Commission of the European Communities' Council Directive 83/467/EEC, 20 rats may be sufficient for an appropriate LD50 determination. LD50 values are often given as milligram of toxicant per kilogram of body weight of the test animals, assuming that twice as big a dose is necessary to kill an animal of double weight. It is therefore easier to compare toxicity data from different species, life stage, or sex. The LD50 values or the related values should not be taken as accurate figures owing to the intrinsic nature of these parameters, as well as the difficulties of determination. Even if you know the exact LD50 value, for example, of parathion to mice (LD50 =12 mg/kg according to *The Pesticide Manual*), and give these doses to a group of animals, for instance, n = 10, the probability that r = 5 will die is only P = 0.246. This can easily be calculated from the binomial formula. However, you can be confident that between 1 and 9 will die (P = 0.998). LD50 values are therefore very useful if you do not need to know the exact number of fatalities, but merely want to describe the toxicity of a compound by one figure. Complicated statistical methods are needed to determine the true confidence limits of LD50. Many statistical methods are described in the books of Finney (1971) and Hewlett and Plackett (1979). Data programs may be used, e.g., Sigmaplot® or Graphpad Prism®. A simple program in BASIC is available (Trevors, 1986), whereas Caux and More (1997) describe the use of Microsoft Excel®.

Table 2.1 shows how toxicants are classified according to their LD50.

Table 2.1 Common Classification of Substances

Toxicity Class	LD50 (mg/kg)	Examples, LD50 (mg/kg)
Extremely toxic	Less than 1.0	Botulinum toxin: 0.00001 Aldicarb: 1.0
Very toxic	1–50	Parathion: 10
Moderately toxic	50–500	DDT: 113–118
Weakly toxic	500–5000	NaCl: 4000
Practically nontoxic	5000–15,000	Glyphosate: 5600 Ethanol: 10,000
Nontoxic	More than 15,000	Water

2.2.5 Acute and chronic toxicity

An important distinction has to be made between acute and chronic toxicity.

Substances that are eliminated very slowly and therefore accumulate if administered in several small doses over a long time may, when the total dose is large enough, cause symptoms. A good example is cadmium that accumulates in the kidneys. Another example is organophosphates that in repeated small doses eventually inhibit acetylcholinesterase more than 80%, which will produce neurotoxic symptoms. Because the inhibition is partly irreversible, many small doses may cause poisoning even though the poison itself does not accumulate. Other poisons (e.g., ethanol) may be given in large, but sublethal doses for years before any sign of chronic toxicity is observed (liver cirrhosis), whereas the acute toxicity results in well-known mental disturbances. In many cases, acute or subacute doses may give chronic symptoms or effects many years after poisoning (cigarette smoking and cancer) or effects in the following generation (stilbestrol may give vaginal cancer in female offspring at puberty).

We use the following terms:

Acute dose — The dose is given during a period shorter than 24 h.
Subacute dose — The doses are given between 24 h and 1 month.
Subchronical dose — The doses are given between 1 and 3 months.
Chronical dose — The doses are given for more than 3 months.

These terms apply to mammals, whereas the times are shorter for short-lived animals or plants used in tests. The dose of a pesticide toward a pest will usually be acute, whereas the dose that consumers of sprayed food will be exposed to is chronic.

2.3 Interactions

One toxicant may be less harmful when taken together with another chemical. If we use *blindness* as an endpoint for methanol poisoning, then whisky

or other drinks that contain ethanol would reduce the toxicity of methanol considerably. When ethanol is present, methanol is metabolized more slowly to formaldehyde and formic acid, which are the real harmful substances. Ethanol is therefore an important antidote to methanol poisoning. Malathion is an organophosphorus insecticide with low mammalian toxicity, but if administered together with a small dose of parathion, its toxicity increases many times. This is because paraoxon, the toxic metabolite of parathion, inhibits carboxylesterases that would have transformed malathion into the harmless substance malathion acid. In another example, a smoker should not live in a house contaminated with radon. Although smoking and radon may both cause lung cancer on their own, smoke and radon gas interact and the incidence will increase 10 times or more when smokers are exposed to radon. (Radon is a noble gas that may be formed naturally in many minerals. It may penetrate into the ground floor of houses and represents a health hazard.)

Two or more compounds may interact to influence the symptoms in an individual and change the number of individuals that get the symptoms in question. Interaction may be caused by simultaneous or successive administration.

2.3.1 Definitions

It is important but difficult to give stringent definitions of various types of interactions or joint action. Because the dose–response curve seldom is linear, and because the relative response to one or more substances given either alone or together cannot exceed 1, we cannot define additive interaction as cases where $p_{(a+b)} = p_a + p_b$.

This is often erroneously done. $p_{(a+b)}$ here is the relative response of two substances A and B, given together in doses a and b, while p_a and p_b are the expected relative responses when a and b are given separately. In cases where there are no interactions, but a joint action, i.e., the animals are exposed to two toxicants at the same time but they act independently, the organisms are killed by one or the other and the relative response may be

$$p_{(a+b)} = p_a + p_b - p_a \times p_b$$

Additive interaction is better defined as cases when half of the LD50 doses of A and B (i.e., LD50(A)/2 + LD50(B)/2) kills 50% when given together. As an example, we can use Parathion oil® and Bladan® and suggest that they have LD50 values of 12 and 10 mg/kg, respectively. A dose consisting of 6 mg/kg of Parathion oil and 5 mg/kg of Bladan will then kill 50%. (The two products have the same active ingredient — parathion.) If more than 50% are killed by such mixtures, we have a case of potentiation, or superadditive joint action, and if fewer are killed, we have antagonism, or subadditive joint action. If one substance is nontoxic alone, but enhances the toxicity of another, we have synergism, and if it reduces the toxicity of

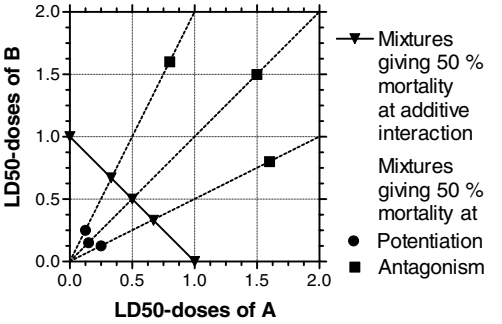


Figure 2.5 Isobolograms showing mixed doses giving 50% mortality in cases of additive interaction, potentiation, and antagonism. When given alone, LD50 = 1 unit for both substances.

the other, we have antagonism or an antidote effect. Endpoints other than 50% deaths may be used in similar considerations. The easiest way to test for interactions and define the various types of interactions is by making an *isobole diagram* (Figure 2.5).

2.3.2 *Isoboles*

Bolos (βολοσ) is a Greek word and may be translated as "a hit." *Isobole* may be translated as "similar hits." When making an isobole, we determine various mixtures of doses of A + B that together give the decided response, for instance, 50% kill. Many different mixtures should be tested in a systematic manner. The compositions of the mixtures given the wanted response are plotted in a diagram where the amount of (A) is given by the y-axis and the amount of (B) by the x-axis.

A typical experiment, where we want to see how A and B interact, using LD50 as the endpoint, may be carried out as follows. The LD50 values of each of the two substances are first determined. A mixture with the same relative proportion as LD50 values is made, e.g., 10 × LD50 units of each. A dilution series is made and the LD50 of the mixture is determined. Dilution series of mixtures with, for instance, 14 × LD50(A) + 7 × LD50(B) and 7 × LD50(A) + 14 × LD50(B) may also be tested. The compositions of the dilution series are marked with three dotted lines, and the compositions of the mixtures giving 50% kill are plotted as points in the diagram.

The location of the points is then compared to the location expected for mixtures with additive interaction, which is the straight diagonal line between points for A alone or B alone (e.g., $LD50_A$ and $LD50_B$). If the points fall outside the triangle, we have antagonism, whereas when inside, we have potentiation.

If one substance is nontoxic but modifies the toxicity of another substance, we get isoboles, as shown underneath. In this case, (B) is nontoxic but functions as a synergist or antagonist to (A).

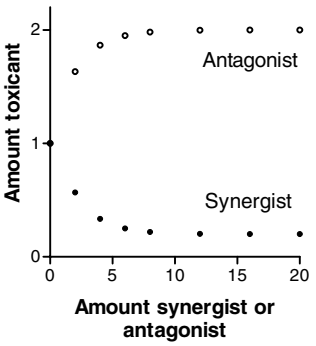


Figure 2.6 The composition of mixtures giving 50% kill in the case of synergism and antagonism when one substance is nontoxic.

The points in Figure 2.6 show isobolograms of mixtures giving 50% kill in the case of synergism and antagonism when one substance is nontoxic. The most important kind of interaction in pesticide toxicology is synergism, and piperonyl butoxide is the most widely used synergist. It inhibits the CYP enzymes in insects that are important for the detoxication of the pyrethrins, many carbamates, and other pesticides. By itself it has a low toxicity to insects or mammals, but its presence increases the toxicity of many pesticides toward insects. In some cases it also reduces the toxicity.

2.3.3 *Mechanisms of interactions*

When two substances react together chemically and the product has a different toxicity to the reactants, we have *chemical interaction*. A good example is poisoning with the insecticide lead arsenate ($PbHAsO_4$), which can be treated with the calcium salt of ethylenediaminetetraacetate and 2,3-dimercapto-1-propanol. These two antidotes react with lead arsenate and make less toxic complexes of lead and arsenate. The antidote atropine works through *functional interaction*. It blocks the muscarinic acetylcholine receptors and thus makes poisoning with organophosphates less severe. Another type of interaction is that one compound modifies the bioactivation or detoxication of the other. CYP enzymes may be induced or inhibited, the depots for glutathione may be depleted, or the carboxylesterases may be inhibited or kept busy with substrates other than the toxicant.

2.3.4 *Examples*

2.3.4.1 *Piperonyl butoxide*

Parathion and other phosphorothionates must be bioactivated to the oxon derivatives in order to be toxic. This is mainly done by the CYP enzymes

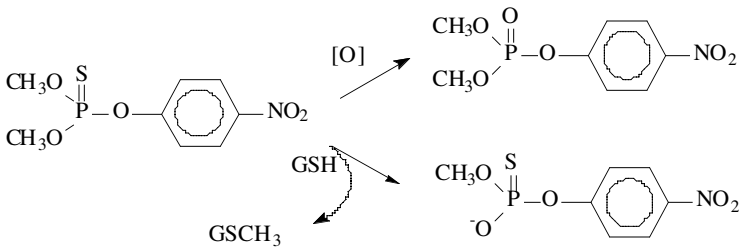
Table 2.2 Effect of Piperonyl Butoxide and SKF 525A Pretreatment on Organophosphate Insecticides' Toxicity in Mice

	24-h LD50 (mg/kg)		
Insecticide	Control (corn oil, 1 h)	Piperonyl Butoxide (400 mg/kg, 1 h)	SKF 525A (50 mg/kg, 1 h)
Parathion-methyl	7.6	330	220
Ethyl parathion	10.0	5.5	6.1
Azinphos-methyl	6.2	19.5	11.8
Azinphos-ethyl	22.0	3.4	9.1

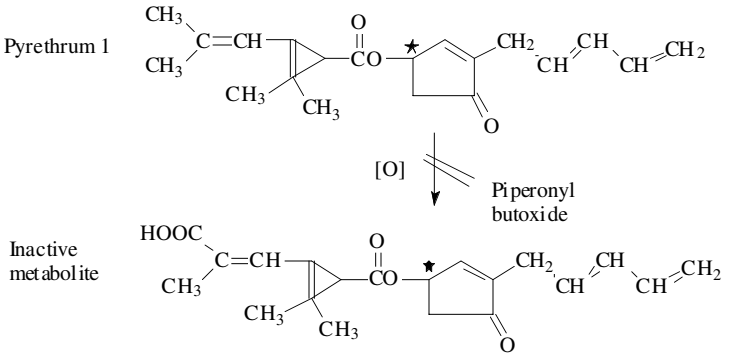
Source: Based on data from Levine, B. and Murphy, S.D. 1977. *Toxicol. Appl. Pharmacol.*, 40, 393–406.

described later. Inhibition of the CYP enzymes with piperonyl butoxide or SKF 525A should therefore reduce the toxicity of parathion and other phosphorothionates. However, experiments with mice show that this is not the case. The symptoms and the time of deaths are delayed, but probably due to other oxidases (e.g., lipoxygenases); the same amount of paraoxon as in the control is gradually formed, only more slowly. Pretreatment with either of the two synergists increases the toxicity of parathion and azinphos-ethyl, but the two CYP inhibitors dramatically reduce the toxicity of the parathion-methyl. A similar pattern was shown for the two azinphos analogues (Table 2.2). The reason for this is that the methyl analogues have a fast route for detoxication through demethylation and therefore need quick bioactivation. If bioactivation is delayed, the detoxication route will dominate.

The involved reactions for parathion-methyl are



The oxidation, which is the bioactivation reaction, is inhibited by piperonyl butoxide, whereas the demethylation reaction catalyzed by glutathione transferase is not inhibited. Piperonyl butoxide is therefore an antagonist to methyl-parathion, but a synergist to most other pesticides, including carbamates and pyrethroids. Pyrethrins are very quickly detoxified by oxidation of one of the methyl groups, catalyzed by the CYP enzymes.



2.3.4.2 *Deltamethrin and fenitrothion*

Sometimes interactions may be detected even when an exact mechanism is unknown. As an example from real life, we can look at locust control in Africa.

Locust (*Locusta migratoria*) is an important pest in Africa. In order to find a suitable pesticide or pesticide mixture, fenitrothion or deltamethrin was tried alone or in combinations by B. Johannesen, a Food and Agriculture Organizaton (FAO) junior expert working in Mauritius. Dilution series of mixtures with different compositions were made and the LD50 values of these mixtures were determined. These values were plotted as shown in Figure 2.7. We see that the two pesticides potentiate each other.

The LD50 of deltamethrin alone was 1.2 μg/g of insects, whereas fenitrothion had an LD50 of 3.5 μg/g of insects. It is shown that the LD50 of mixtures of various compositions is lower than expected in cases of additivity. Hundreds of insects were used to determine the plotted LD50 doses of the mixtures. The great scatter illustrates the inborn uncertainty of such determinations. All the points are well inside the line for additivity, and some kind of potentiation is evident.

2.3.4.3 *Atrazine and organophosphate insecticides*

Sometimes more surprising examples of interaction may be observed.

The herbicide atrazine is not toxic to midge (*Chironomus tentans*) larvae but has a strong synergistic effect on several organophosphorus insecticides such as chlorpyrifos and parathion-methyl, but not to malathion. The increased rate of oxidation to the active toxicants, the oxons, is suggested as one of the mechanisms, and the level of CYP enzymes is elevated. Figure 2.8 shows the effect of the herbicide atrazine on the toxicity of chlorpyrifos. The data from Belden and Lydy (2000) show typical synergism. Altenburger et al. (1990) and Pöch et al. (1990) have described other examples of the use of isobolograms and how to interpret them.

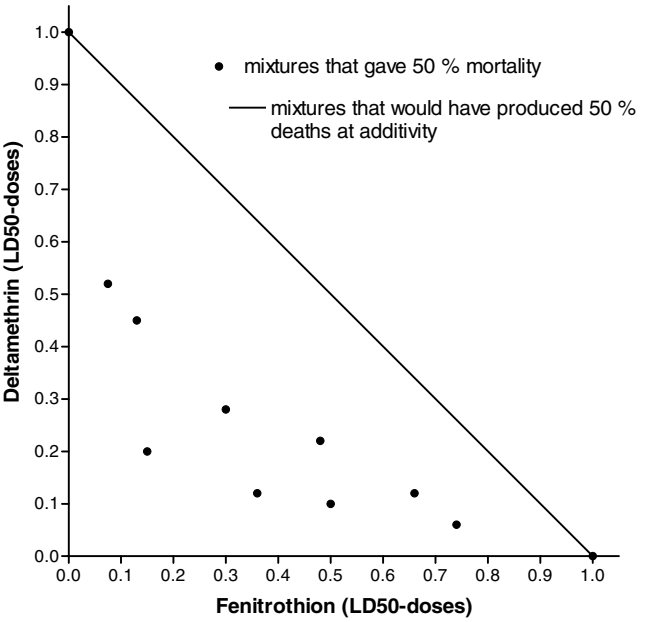


Figure 2.7 An isobologram of *Locusta migratoria* given mixed doses of deltamethrin and fenitrothion. Given separately, an LD50 dose of deltamethrin is 1.2 μg/g and of fenitrothion is 3.5 μg/g. The figure is based on data provided by Baard Johannessen and will be later published in full text.

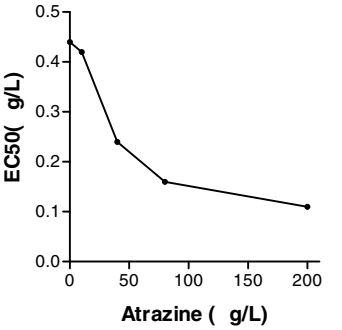


Figure 2.8 The effect of atrazine on the toxicity of chlorpyriphos. (Data from Belden, J. and Lydy, M. 2000. *Environ. Toxicol. Chem.*, 19, 2266–2274.)