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The more specific we are, the more universal something can become. Life is in the details. If you generalize, it doesn't resonate. The specificity of it is what resonates.
Jacqueline Woodson

Bench Marks!

Chemical Concentrations in Cell Culture Compartments (C5) – Concentration Definitions

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Abstract

Some laboratory issues are taken for granted as they seem to be simple and not worth much thought. This applies to "concentrations of a chemical tested for bioactivity/toxicity". Can there be any issue about weighing a compound, diluting it in culture medium and calculating the final mass (or particle number)-to-volume ratio? We discuss here some basic concepts about concentrations and their units, addressing also differences between "dose" and "concentration". The problem of calculated nominal concentrations not necessarily corresponding to local concentrations (relevant for biological effects of a chemical) is highlighted. We present and exemplify different concentration measures, for instance those relying on weight, volume, or particle number of the test compound in a given volume; we also include normalizations to the mass, protein content, or cell number of the reference system. Interconversion is discussed as a major, often unresolved, issue. We put this into the context of the overall objective of defining concentrations, i.e., the determination of threshold values of bioactivity (e.g., an EC50). As standard approach for data display, the negative decadic logarithm of the molar concentrations (–log(M)) is recommended here, but arguments are also presented for exceptions from such a rule. These basic definitions are meant as a foundation for follow-up articles that examine the concepts of nominal, free, and intracellular concentrations to provide guidance on how to relate *in vitro* concentrations to *in vivo* doses by *in vitro*-to-*in vivo* extrapolation (IVIVE) in order to advance the use of new approach methods (NAM) in regulatory decision making.

Concentrations do matter – don't they?

Many pharmacology and toxicology books start with the famous Paracelsus quote from the 16th century that "*All things are poisons, for there is nothing without poisonous qualities. It is only the dose which makes a thing poison*". In more modern terms, referring to *in vitro* methods, this quote may be modified to: "*all chemicals are toxic, for there is nothing that does not disrupt cell function if its concentration is high enough. It is only the concentration that makes a thing a toxicant*". Is this really so? Yes, indeed: water will kill cells, kitchen salt (NaCl) will kill cells, and also sugar will kill cells, if high enough concentrations are chosen. On the other hand, cells can live, proliferate, and function happily in the presence of cyanide, anthrax or botulinum toxins, strychnine, tetrodotoxin (TTX), methylmercury or E605 (parathion) and VX (a dual component

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chemical weapon), i.e., some of the deadliest poisons known. Viability will remain unaffected if the concentrations are below the toxic threshold (Krebs et al., 2018). Thus, concentrations do obviously matter, and this seems to be a self-evident, apparently trivial fact. In practice, it is therefore astonishing how many problems are found in the scientific literature concerning the concentration concept. Of particular pharmacological-toxicological interest are the following three areas:

1. How do concentrations affect specificity?
This topic will not be addressed here, but it is of eminent general importance. Compounds are quoted as being hepatotoxicants, neurotoxicants, reproductive toxicants, etc., but this apparent specificity applies only to certain concentrations (frequently with less than an order of magnitude between specific target organ and other effects, or specific effects only due to accumulation in certain tissues). Similarly, compounds are classified as specific receptor antagonists, apoptosis inducers, antioxidants, or triggers of other biological functions (Gerhardt et al., 2001; Hansson et al., 2000; Latta et al., 2000) although only few chemicals show two or more orders of magnitude between specific and unspecific effects. Nevertheless, a screen of the literature shows that drugs, pesticides, and environmental agents are often used at concentrations more than orders of magnitude higher than their specificity threshold, or the threshold known to trigger human effects.

2. How is general information derived from concentration-response data?
In a large number of experiments, information is not sought for one particular concentration. Instead the question is more about defining a threshold concentration in a way that, e.g., concentrations above are considered toxic or bioactive, while concentrations below are considered inactive. Such threshold data can then have far-reaching implications, such as their use to define safe working concentrations or safe levels in foods. Such threshold values are compound-specific, and they can span at least 6 orders of magnitude. Note that pM concentrations of botulinum neurotoxin can be toxic, while high µM concentrations of TTX are tolerated by nearly all cells (however, some cell functions may be incapacitated by nM concentrations of the same compound!); some compounds, such as DMSO or mannitol, can even be beneficial for some cells in the mM range, while many other chemicals would kill in this concentration range. Therefore, concentration thresholds have to be carefully derived for each given chemical. In order to set up reliable methods for this, there needs to be basic agreement on the definition of concentrations as such (see below), and on the interconversion of such information from one system (e.g., animals or cell cultures) to another (man). Moreover, analytical measures to quantify concentrations need to be established and validated (a topic not covered here).

3. How are concentrations defined?
This last question is at the core of this article (and an essential basis for areas 1 and 2, which are not covered here explicitly), and we will approach it stepwise.

Key questions indicating the complexity of "concentrations"

We claim that the handling and definition of concentrations is less trivial than it often appears, even if issues of specificity and problems of deriving threshold values are left aside. The complexity of the concentration concept becomes obvious when we try to answer the three following questions:

1. How can one define, interconvert, and compare concentrations in a tissue (cell aggregate), a body fluid, and a cell culture medium?
2. Is it the dose (amount of compound per cell culture compartment), the cell dose (the amount of compound available per cell) or the concentration (amount of compound per volume unit) that determines the toxicity threshold?
3. Is the nominal concentration of a compound a meaningful measure at all? *Note that we must assume (without usually verifying this) that the compound was pure and intact before stocks were made, that it is stable in stocks and cell culture medium, that it distributes evenly, and that it does not bind to cell culture constituents.*

Below, and in follow-up articles, we will discuss some of these questions, without aspirations to cover the topic comprehensively. Many excellent papers have dealt with these topics, and only few can be mentioned (Gülden et al., 2001, 2002; Kramer et al., 2012, 2015; Bessems et al., 2014; Bosgra and Westerhout, 2015; Armitage et al., 2014; Doskey et al., 2015; Groothuis et al., 2015; Fischer et al., 2017; Wambaugh et al., 2018; Casey et al., 2018; Bell et al., 2017). Here we attempt to make the topic accessible for a non-specialist community. This series of C^5 articles is intended to provide some basic insight as well as some handy tools that may be used immediately, without further prior knowledge, and not requiring extensive training.

What is a concentration?

There are some basic issues about concentrations that must be considered to transparently describe methods and data (Fritsche, 2015; Bal-Price et al., 2018; Leist and Hengstler, 2018). The most fundamental question is the physical quantity chosen to define a concentration. Different disciplines have their traditions and conventions, and little thought is given in the standard literature to these divergent approaches and the problems of interconversion. In all fields, a "concentration" is considered to be *the ratio of (i) chemical and (ii) the surrounding system*. The chemical may be expressed in terms of its volume, its mass, the number of its molecules/particles, or in terms of physical or biological properties such as its radioactivity. The system may be expressed as weight or volume. Alternatively, it may be quantified by the number or mass of certain molecules in the system (e.g., water, water-free substance, protein, DNA, or lipid). Thus, *there are more than 10 common ways to define the term "concentration"*.

The interconversion of these measures is not self-evident and often requires both background knowledge and additional information. For instance, if concentration is given in ppm (parts per million), background information is required on what is meant

by parts (molecules, weight or volume) and what method is used to quantify such measures (for the system this is mostly not an analytical method but an assumption/historical value). Even more complicated are typical measures of tissue concentrations *in vivo* (applying also to *in vitro* microphysiological systems) (Hartung and Leist, 2008; Alépée et al., 2014; Gordon et al., 2015; Marx et al., 2016): they may be given "per g wet weight", "per g dry weight", "per protein content", "per lipid content", etc. For *interconversion of such measures*, e.g., to molarity, background information is required on the protein concentration (for "per protein content") or on the water-free mass per volume unit (for "per g dry weight").

Many *in vivo* studies (but also *in vitro* experiments) are performed with radioactively-labelled compounds. In such cases, the chemical is quantified in terms of radiation released, and concentrations may be given in "radioactivity per gram wet weight" (using a unit of Bq/g). Here, at least background information on the specific activity of the labelled chemical is required. Notably, this knowledge is often not sufficient, as metabolism of the chemical may occur (and radioactivity may end up in the breakdown products), or the time-point of measurement may play a role (for short-lived activities). Such complexities are not unique to radioactivity; they essentially apply to all cases, where a chemical is quantified indirectly on the basis of an "activity", e.g., hormones, cytokines, enzymes, and toxins.

What is a dose?

From the above, it is clear that comparability of concentrations is not a trivial issue, and that it is essential to define such issues for multi-partner projects and collaborative activities. It certainly is also beneficial in terms of scientific communication in general. In this context, some fundamental decisions need to be taken on whether "amounts of chemical" in a system are to be expressed as concentrations at all. Is there an alternative to this? Yes, there is, and it is very important to be aware of this to avoid confusion and mistakes. Instead of characterizing the chemical quantity as a ratio of chemical and system (relative measure), the rationing may be omitted (absolute measure). The latter approach leads to the concept of "a dose". *A dose is a measured quantity of a chemical delivered to a system*. The greater the quantity delivered, the larger the dose. This concept is easiest understood for medicine. The unit is the human body, and the dose is the amount (usually weight) of chemical delivered. A typical dose is packaged in a tablet. For instance, 500 mg of aspirin is taken as a dose (for headache) or 10 mg zolpidem is taken as a dose (to promote sleep). The same applies to animals. For instance, a dose of 50 mg praziquantel may be given to a dog to eliminate parasites. Notably, a dose is the total amount delivered.

However, doses can also be normalized to the size or the weight of the unit. For instance, *normalized doses* can be indicated in amount of chemical per kg body weight. In the case of the dog, one could specify 5 mg praziquantel per 1 kg of body weight of the dog. In pharmacological and toxicological research, normalized doses are common to improve comparability across individuals and across species. However, this is rarely applied to human patients in daily practice, e.g., patients between 50 and 150 kg body weight may be given the same dose of medication for blood pressure or asthma. Exceptions are, e.g., drug doses for children *vs* adults, or drugs with a narrow therapeutic index (anti-cancer drugs, some anti-epileptics, etc.). However, the normalization to body weight is a relatively coarse approach with multiple shortcomings, and other normalizations are also common (e.g., "per body surface area", or "per lean body mass"). Notably, normalized doses, are not to be confused with concentrations.

Concentrations and normalized doses sound like related concepts. If they are only different modes of describing similar experimental conditions (physical measures), then the interconversion should be relatively easy. However, the concepts neither overlap, nor is interconversion of the measures trivial. It is in fact virtually impossible to convert doses to concentrations (and *vice versa* for that matter), unless a large amount of background information is available, and several assumptions on experimental conditions are made. Some approximation methods to predict concentrations from normalized doses will be discussed later in this series.

For the application of non-animal approaches, the concept of dose needs to be translated to cell cultures. In this case, there is no clear (generally accepted) definition of what the reference unit is. The two most common ones are "a cell" and "a cell culture dish". Thus, *the dose may relate to the amount of chemical added to a cell culture dish*. As the conditions in such a dish are highly defined, the normalized dose appears similar to a concentration, and the term "*in vitro* concentration-response curve" is frequently found in the literature. Is there really a meaningful difference between dose and concentration in cell cultures? Some examples can best clarify this:

(1) If 1000 cells are placed in a cell culture dish in 1 ml of medium, and 1 mg chemical is added, then the dose is 1 mg/dish or 1µg/cell; the concentration is 1 mg/ml. If the same cells are cultured in 2 ml medium, and the same amount of compound is added (1 mg), then the dose is still 1 mg/dish and 1 µg/cell; however, the concentration is 0.5 mg/ml.
(2) Imagine an experiment to investigate nanoparticle (NP) toxicity: one laboratory uses 1000 cells in 0.1 ml of medium; another laboratory has a different test format, culturing the 1000 cells in 1 ml of medium. They both agree to use 100,000 NP/ml. In the first lab, the cells will be exposed to 10 NP/cell. In the second lab, cells will face 100 NP/cell.

The examples show that concentrations and normalized doses are different concepts. But they also indicate that the interconversion of a relative dose to a concentration is straightforward and un-complicated *in vitro* (relative to the large problems *in vivo*). Strictly speaking, using the term "dose-response" for new approach methods (NAMs) is usually incorrect, as concentration-responses are meant. Although intervonversions are relatively easy, the distinction is still meaningful and relevant for the interpretation of data.

Are cells killed by concentrations or by doses?

The goal of many experiments in quantitative biology (biochemistry, physiology, pharmacology, toxicology, etc.) is to define a

threshold "amount" of chemical that leads to a biological effect. For example, such effects may be cell death, activation of a signaling pathway, or labeling of a structure. Back to the above issue: Is the right measure of the quantity of chemical a concentration or a dose? Unfortunately, there is no generally correct answer. It depends...

In the majority of cases, *concentrations are the more relevant physical quantity* and it is most common to define biological effects of chemicals *in vitro* in terms of concentrations triggering the effect. The situation is different *in vivo*. Here, threshold effects are usually given in categories of doses or normalized doses. The reason is that the dose is usually easy to measure or to define, while concentrations *in vivo* are an extremely complicated concept, and the data vary continuously with time and space (location coordinates within a body). The variable ease of access and knowledge on *the physical quantities dose* vs *concentration, and the resultant different use of these quantities* is the basis of the fundamental problem of converting reference values from *in vivo* to *in vitro*, and *vice versa*.

Why are concentrations the most relevant measure *in vitro*?

Most effects of chemicals are assumed to be mediated by an interaction of a chemical with a biological target, e.g., a receptor or a stretch of DNA. Many chemical-target interactions are non-covalent/reversible, and they can be described by *the law of mass action*. This law is based, contrary to what may be deduced from its ancient name, on concentrations of chemicals. Thus, in most cases, the mathematical description of the bioactivity of a chemical is best based on concentrations.

When is this not the case? From the above, one may deduce that problems may arise when reactions are irreversible, or when a target becomes saturated so that equilibrium conditions are not possible. Problems with the concentration concept also may be found in situations where a chemical does not distribute evenly in a cell culture dish (and thus the average nominal concentration is not representative of the target concentration). Adsorption to plastic or accumulation in cell compartments may play a role here. Another non-equilibrium condition that provides problems for the concentrations concept is instability of a chemical, so that the time (not considered in the law of mass action) plays a role. The following *examples show cases, where the dose of a chemical needs to be considered to describe* in vitro *effects:*

- In many cell cultures, hydrogen peroxide (H_2O_2) is eliminated rapidly by the cellular catalase. If there are more cells (more catalase), H_2O_2 has a shorter half-life and thus cells are exposed for a shorter time to toxic concentrations. At higher doses (but similar concentrations), the elimination of H_2O_2 takes longer, and thus makes a given concentration appear more toxic. Therefore, it has been suggested to indicate H_2O_2 toxicity in terms of cell dose (e.g., µmol H_2O_2 per 1000 cells) (Gülden et al., 2010).
- Dopaminergic neurons have a high capacity to import the neurotoxicant methyl-phenylpyridinium (MPP+). The same is observed in the dopaminergic cell line LUHMES (Schildknecht et al., 2009, 2015, 2017; Efremova et al., 2015). The import of the toxicant through the dopamine transporter is so efficient that a large portion of the cell culture medium is cleared of MPP+, and this can lead to a limitation of the toxicity if the overall amount (dose in the cell culture dish) is not sufficient to fill all cells with sufficient toxicant to affect cellular respiration (Pöltl et al., 2012; Terron et al., 2018). Thus, the overall dose of MPP+ can play a role, in addition to its concentration.
- The same applies to other toxicants that are strongly accumulated in cells, be it by active mechanisms (transporter-dependent, membrane potential-dependent) or by passive mechanisms (high affinity binding to cellular structures; accumulation in lipophilic compartments; covalent interactions). A typical example is methylmercury chloride. This lipophilic compound accumulates in cells and binds covalently to sulfhydryl groups. Therefore, cellular concentrations can be 100 times higher than the medium concentration (Zimmer et al., 2011a, 2014; Aschner et al., 2017). Taxol is an example of cellular accumulation due to high affinity binding (to cellular microtubules) (Volbracht et al., 1999, 2001). For highly hydrophobic compounds, such a dieldrin, accumulation on cell culture surfaces and in cellular membranes may play an important role. Accumulation, and thus depletion from the cell culture medium, may also play a role for some dyes (DNA intercalators, membrane stains), toxins, and compounds trapped by intracellular metabolism (acetoxymethylesters).

At present, there is no ideal solution to the issue that the nominal concentration correlates well with the bioactivity for some but not all compounds, and dose plays an additional (or sometimes even pivotal) role. The most common procedure is to generally refer to concentrations, and a majority of chemicals dissolving well in cell culture media will be described sufficiently well in this way. The overall *usefulness of using nominal concentrations to characterize bioactivities* is reflected by the many toxicological/pharmacological parameters that refer to concentrations. They include the IC50 (median inhibitory concentration), the EC50 (concentration evoking 50% of the maximal effect), the HNCC (highest non-cytotoxic concentration), C_{max} (peak plasma concentration), AUC (integral of the concentration time curve), KD (dissociation constant for a reversible binding process, indicated in concentration units), or the MAC (minimum alveolar concentration required for inhalation anesthesia), and many more.

Upon a more detailed analysis of the concentration concept (discussed in a later part of this series), it will become evident that so-called nominal concentrations (mathematically-determined, theoretical average concentrations in a cell culture dish) do not describe the respective target site concentrations. When methods are applied to better define the true concentrations, they correct for some of the distribution problems mentioned above, and the approach to refer to doses becomes less necessary to define thresholds at which chemicals affect biological functions and structures.

Suitable measures of concentrations

If, and when, concentrations are measured to derive important summary and threshold data, then an important practical question arises: What is the most useful quantity definition, i.e., which type of unit should be used? The first type of consideration concerns the type of unit, i.e., the preferred physical measure, such as fraction (ppm), weight per volume (mg/ml), or molarity (particles per volume; mM). In toxicology and pharmacology, there is a strong case for using molarity, as this has a clear definition (as opposed to ppm), does not require additional background knowledge/information, and allows direct comparison of numbers (as opposed to weight/volume units).

- In a test reacting specifically to lithium ions, the threshold molarity would be the same for the chloride, mesylate, or iodide salts, and it would be independent of whether the crystals contain two or six water molecules per unit. If, however, the threshold value is given in µg/ml, it will be different for all these ways to solubilize lithium ions.
- If one compares the toxicity of polychlorinated biphenyls in weight/volume units, then the highly chlorinated (high molecular weight) congeners will always appear to be less toxic, while expression of the EC50 in molarity compares the same numbers of molecules, and thus is scientifically more appropriate (Nyffeler et al., 2018).
- For screening, often an upper level (a highest screened concentration) is defined (Nyffeler et al., 2017; Delp et al., 2018). For instance, all compounds are screened up to a concentration of 50 µM or up to 50 µg/ml. In the latter case (50 µg/ml), one would screen low molecular weight compounds at much higher molar concentrations than high molecular weight compounds. This would lead to a misrepresentation of the bioactivities.

So, *what are reasons to still use weight/volume measures*? For most applications, there is no good reason besides tradition and practical convenience. Convenience refers to the issue that preparation of weight/volume stock solutions is always possible, even without knowing molecular weight or the purity of a compound. Thus, such approaches are well justified for undefined extracts, mixtures of compounds, substances of unknown molecular weight, etc. They must be employed in cases in which molarity cannot be calculated. However, it is hard to understand why this approach would be transferred to areas where exact information on the molecular weight and compound purity is available, and thus molarities can be calculated.

In some fields, an alternative to the use of molarities is the use of *unitless fractions* (%, ppm, ppb, etc.). These may be useful for non-aqueous systems, for which the use of molarities is uncommon, e.g., mixtures of gases or solvents, contaminants in metals and chemicals within solid feed (used for feeding studies in toxicology). The practical problem here is that it is not evident whether the ratios refer to volumes, weights or particle numbers, and different fields use different rules and definitions. It is clear that for most experimental systems (combination of a chemical with its background substance) a volume percentage will not be the same as a weight percentage, and thus these units cannot be used unambiguously without additional background information.

In biologically very complex situations (mostly referring to endogenous metabolites such as NADH, enzymes or RNA molecules) yet another approach is taken: concentrations are not given as absolute measures, but only as relative changes compared to a control/normal situation (Kuegler et al., 2010; Zimmer et al., 2011b; Efremova et al., 2015; Nyffeler et al., 2018).

If one has indeed decided on the use of molarity to characterize the activity of chemicals, is there any *guidance on the use of the unit* (e.g., nM, µM, mM)? There is no scientific reason to prefer one over the other. However, there may be practical issues, e.g., related to data display. For instance, if compounds act in the µM range, indication of the numbers in mM is inconvenient, as many zero digits need to be included, and the same applies *vice versa*. When a larger range of values is covered, logarithmic scaling is useful. For instance, if all values are given in log molarity (log(M)), large ranges can be covered (Krebs et al., 2018), and this approach allows keeping numbers in an easy (convenience for human users) format with one pre-comma digit. However, all numbers will be negative (one µM will be -6, one mM will be -3). It may be inconvenient to use negative numbers throughout. For this reason, the use of -log(M) is common for large chemical screens in pharmaceutical industry. The latter could be a *universal data format* to be used for most applications. The only disadvantage is that graphical data display usually follows the convention that small numbers are on the left side of the x-axis, and large numbers are right. To follow this tradition, one has to accept that 9 (referring to 1 nM) is a smaller number than 3 and use a data display program that can deal with this condition. Alternatively, one must become accustomed to curves looking different from the usual text-book display (horizontal mirroring).

In summary, the following example compiles the above discussion: In a given cell culture system, the toxicity (EC_{50}) of the three toxicants A, B, and C may be 6.0 (given in -log(M)). This means that a nominal concentration of 1 µM kills or impairs 50% of the cells. If the compounds have molecular weights of 100, 500, and 2500 Dalton, then the toxicity expressed in µg/ml would differ by factors of 5 and 25. Another potential difference may be that the toxicity of compounds A and B may be 6.0, independent of the plate format and cell number used, while the toxicity of C may be 4.0 in another culture dish with higher cell numbers.

Outlook

Three further issues need a future dedicated discussion: (i) the nominal concentration of a chemical may not correlate with its free bioactive concentration in cell culture medium (Blaauboer et al., 2012; Westerhout et al., 2011); (ii) the concentration of a toxicant inside a cell, where the chemical's target is located, may not correlate with its free medium concentration; (iii) the threshold concentration determined *in vitro* may need to be converted to the respective *in vivo* concentration, or possibly to a dose that results in such a concentration. The procedure to predict such

measures from *in vitro* concentrations is called *in vitro*-to-*in vivo* extrapolation (Wetmore et al., 2014; Wetmore, 2015; van Vliet et al., 2014; Bell et al., 2017; Casey et al., 2018; Leist et al., 2010), and it is "the philosopher's stone" to make data from NAM useful for toxicology and pharmacology as disciplines to quantitatively predict chemical effects in man (Leist et al., 2014).

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Conflict of interest

The authors declare that they have no conflict of interest.

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