The relevance of the facilitated transport hypothesis for *in vitro* to *in vivo* extrapolation in toxicology.

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Abstract

Issues concerning free and nominal concentrations of toxic compounds *in vitro* and *in vivo* are widely recognised. When data from *in vitro* experiments is extrapolated to *in vivo* situations it has been understood for a long time that factors like, complex forming and protein binding greatly influence the bio-availability of compounds. The data is normally better explained by extrapolating data using free instead of nominal concentrations in tests. However, it has been shown that in some cases using free concentration results in an under-estimation of the toxic effect. In this study it is reviewed how and to what extent facilitated transport influences the accuracy of extrapolation methods. It will be explained how facilitated transport is defined and how the theory originally designed for metal complexes might be extrapolated to other situations like facilitated transport by proteins. Furthermore it will be discussed that the influence of facilitated transport only has to be taken into account when certain criteria are met. Firstly, there has to be an extracellular ligand for the agent of interest, second the Davison criterium has to be met, thirdly the derived data should be influenced by alterations in kinetics and the ligands should be present or absent in either the *in vitro* or *in vivo* situation.

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Chapter 1. Introduction

1.1 *In vitro* assays in toxicity testing

There is an urgent need for the toxicology testing of drugs, chemicals and cosmetics. The EU's new chemicals legislation, REACH (Registration, Evaluation and Authorization of Chemicals) has a goal of testing and collecting the data of more than 30,000 substances within the next decade. It is estimated that this will be at a cost of at least 4 million animals unless additional alternative validated testing methods become available in the near future (Clemedson, Kolman et al. 2007). The prospect of using millions of additional animal testing is not only unacceptable to the public, it is also expensive (Ackerman and Massey 2004). The 7th Amendment to the cosmetics directive added additional pressure for the validation of alternative testing methods as it called for a total ban on animal testing of cosmetics by 2009, and 2013 for those tests for which alternatives have not yet been found.

There is clearly an urgent need for the replacement of toxicity test on animals in the short run and *in vitro* testing is a favored alternative (Blaauboer and Andersen 2007; Andersen and Krewski 2009). The traditional methods used today for the prediction of human toxicity are based on animal testing. For example he traditional acute toxicity tests used for regulatory purposes are the Organization for Economic Co-operation and Development (OECD) Test Guidelines (TG) 420, 423 and TG 425 (Clemedson, Kolman et al. 2007) and test guidelines on toxicokinetics TG 417. Those are all based on administering toxicants to animals. In the MEIC project in 1998 it was shown that the 24 hour *in vitro* tests on human cell lines predicted human acute toxicity better than test on rats and mice (Ekwall, Barile et al. 1998).

The importance of *in vitro* assays in toxicology therefore is clear. For the various reasons explained above there is an urgent need for toxicity test that are, cheaper faster and scientifically more robust that traditional assays. Furthermore *in vitro* test often offer possibilities that show advantages over traditional animal testing or clearly have the potential of being a more favourable alternative. Tests on cell lines are faster than tests on complete organisms, they are a lot cheaper and the scientific data that can be obtained from tests on cell lines often explains more about toxicological pathways and toxicological endpoint on a cellular level (National Research Council 2007; Andersen and Krewski 2009).

However much research has been carried out and published over the last years on the topic, many more questions are raised. One of the goals to be reached two years ago as described in "Toxicity testing in the 21st century a vision a strategy (2007)" was to obtain further understanding of the relationship between the in vitro data and in vivo data. In other words this can be formulated as, the refinement of the *in vitro* to *in vivo* extrapolation (IVIVE) (National Research Council 2007). There are many issues concerning this topic. the most important differences between *in vivo* and *in vitro* situations involve differences in plasma protein binding and concentration, sediment binding and type of sediment, binding to plastics, direct contact exposures and blood and

system fluid flows (Gülden, Mörchel et al. 2002; DelRaso, Foy et al. 2003; Gülden and Seibert 2005; Bopp, Bols et al. 2006; Nichols, Fitzsimmons et al. 2007; Pelkonen and Turpeinen 2007; Pelkonen, Kapitulnik et al. 2008). Especially since there is a trend of developing complicated models like the Biology based kinetic (BBK) and physiologically based pharmacokinetic (PBPK) models in pharmacology and toxicology. One of the major issues concerns the bio available concentration in *in vitro* assays to determine half maximum effect concentrations (EC50) and median lethal concentrations (LC50), clearance and metabolism rates and other toxicity and toxico-kinetic data.

For the determination of the bio available concentration some simple concepts should be distinguished. It can be said that the concentration to which cells or organisms are exposed is always the nominal concentration. The nominal concentration is the "total" concentration of a compound in any medium described by the total amount of compound in any system divided by the volume of the system. A second way of putting it is to consider the freely dissolved concentration as the bio-available concentration. The freely dissolved concentration is the concentration in the liquid phase to which the cells or organisms are directly exposed. It has been understood for some time now that the nominal concentration in cell culture mediums in *in vitro* assays should not always be used as the concentration to which the cells were exposed when extrapolating the data to *in vivo* situations. However, on the other hand in vivo data are not always described well by using only free concentrations.

1.2 Nominal and free concentrations

Many papers have been published concerning agent concentration in mediums or blood plasma and how this influences pharma- and toxicokinetic modelling. It is now largely accepted that the actual concentration to which cells are exposed should not always be the nominal concentration in blood plasma, cell culture medium or water (Gülden, Mörchel et al. 2002; DelRaso, Foy et al. 2003; Meylan, Behra et al. 2004; Gülden and Seibert 2005; Degryse, Smolders et al. 2006; Grime and Riley 2006; Baker and Parton 2007; Kramer, Van Eijkeren et al. 2007; Pelkonen and Turpeinen 2007; Rodgers, Davis et al. 2007; Andersen and Krewski 2009). Examples of this are the free ion activity model (FIAM) and BBK and PBPK models that take extracellular matrix binding in to account. In these models it is assumed that only the freely dissolved compound is available for uptake into the cell and therefore the only fraction of agent that can cause toxic effects. It is fact that often the major part of compounds is bound to protein, fat content, sediment, dissolved organic matter or inorganic ligands. This fact is mosly considered to only influence free concentrations. It is however sometimes not so straightforward and the assumption that agents bound to another dissolved phase are inactive and do not influence target cell uptake kinetics both *in vivo* and *in vitro* might be wrong (DelRaso, Foy et al. 2003; Degryse, Smolders et al. 2006). Figure 1-1 and 1-2 give a good indication of compound distribution over different phases *in vitro* and *in vivo* respectively.

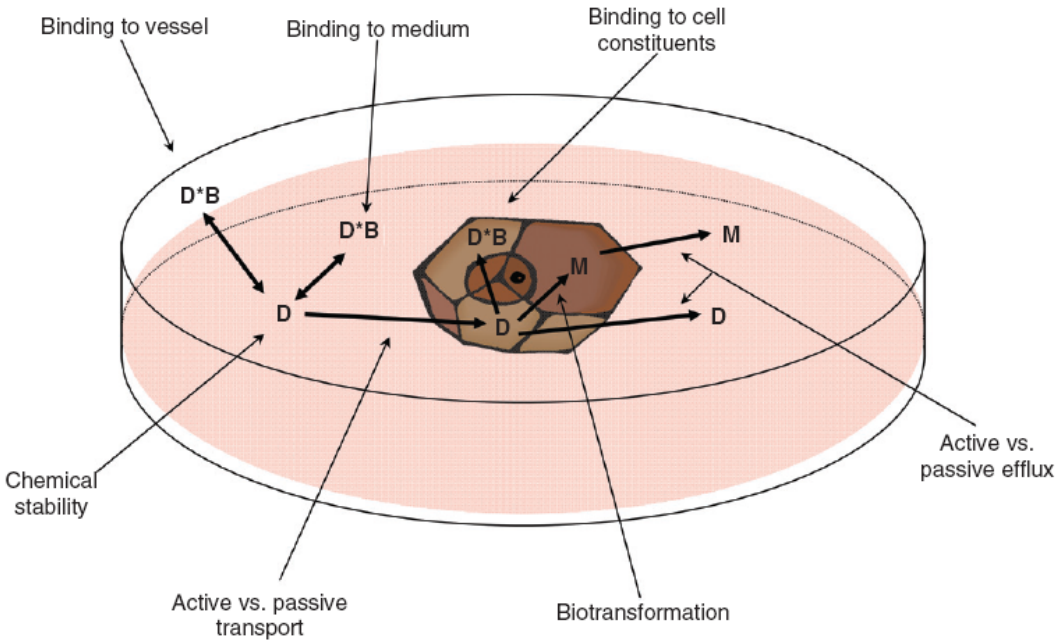

figure 1-1: "Example of an *in vitro* system for estimating hepatic clearance. Various factors affecting the concentration of the compound and its movements and transformation in the *in vitro* system are indicated. D, drug; M, metabolite." (Pelkonen and Turpeinen 2007)

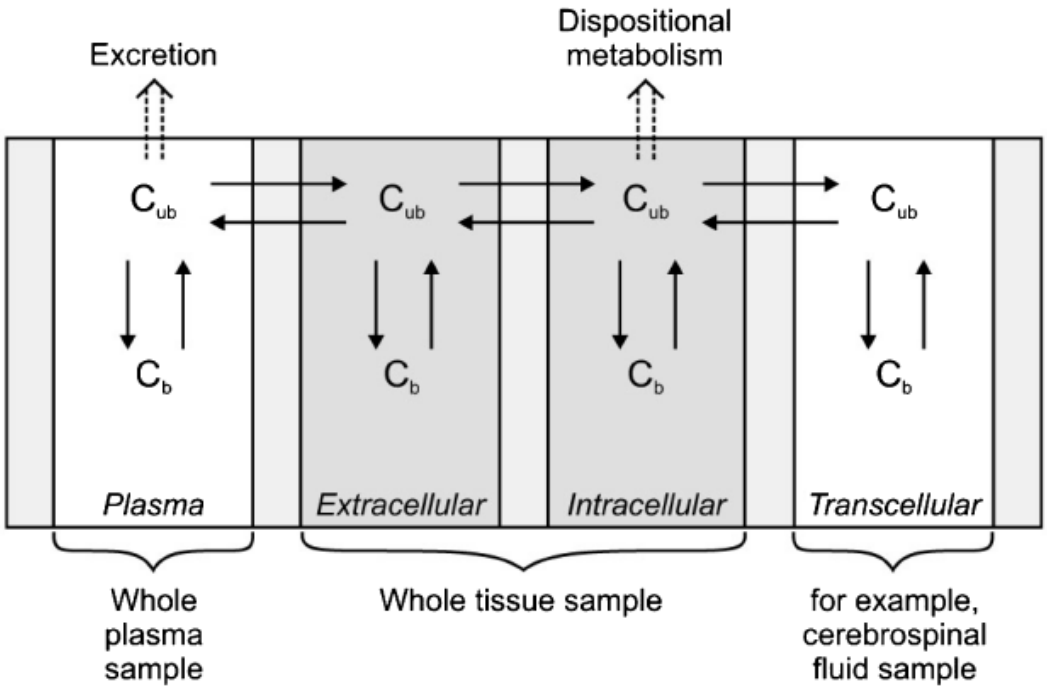

figure 1-2: "Distribution of a drug/chemical between plasma and tissue and the significance of its binding to plasma and tissue components. Transcellular compartments are, e.g., cerebro-spinal fluid or intracanalicular bile. Sampling possibilities and removal of a compound from the system are also indicated." (Pelkonen, Kapitulnik et al. 2008)

1.3 Concentration issues with respect to *in vitro* to *in vivo* extrapolations

When extrapolating data from *in vitro* to *in vivo* situations it is always necessary to make many assumptions about target sites, scaling factor and uptake kinetics (Pelkonen and Turpeinen 2007). Assumptions about biochemical or toxicological mechanistic differences between *in vitro* cell cultures and *in vivo* situations will always have to be made unless all biologic and biochemical mechanisms have been modelled. Therefore it can be expected that some under- or overestimation of toxic effects of agents measured *in vitro* will always remain (Schirmer 2006). However, estimations about concentrations at active site, that are sometimes poorly made, should be relatively simple to predict. (Pelkonen, Kapitulnik et al. 2008). Since processes influencing agent concentrations at active sites are by far not as diverse and complicated as mechanistic issues, it is just a matter of modelling those processes in the right way, in order to make reliable models for active sites concentrations of agents. Many processes have been or are being researched in terms of compound distribution over systems and concentration at target sites both *in vitro* and *in vivo* such as extracellular matrix binding, sediment binding, blood flow issues and many more (Foy and Frazier 2003; Devlin, Frampton et al. 2005; Gülden and Seibert 2005; Van de Waterbeemd 2005; Degryse, Smolders et al. 2006; Grime and Riley 2006; Gülden, Dierickx et al. 2006; Baker and Parton 2007; Nichols, Fitzsimmons et al. 2007; Pelkonen and Turpeinen 2007; Sogorb, Alvarez-Escalante et al. 2007; Pelkonen, Kapitulnik et al. 2008; Andersen and Krewski 2009).

Apart from compound distribution over different phases in equilibrium there is also a kinetic effect. Whenever an agent enters a system the initial distribution of the agent over different phases in the system will not be equal to equilibrium conditions. It will always take some time for a system to reach equilibrium and in this time uptake and complexation kinetics play an important role for modeling of toxico-kinetic processes (this will be explained more clearly in section 2-2 and 2-4) . It is harder and more complicated to measure equilibrium rates and fluxes from one phase to the other than to asses partition coefficients. Normally one can calculate and model those rates and fluxes by using thermodynamic law's like Fick's law (equation 2-1) that describes diffusion from one phase to another (Kramer, Van Eijkeren et al. 2007). However when processes become more complicated due to active transport or other processes the kinetics will often have to be described by more than one term. For example, it is often seen that cell uptake fluxes are described by a term for diffusion (Fick's law) and a term for active transport (Jansen, Blust et al. 2002).

The interest of this study lies in the way passive transport (transport by diffusion) should be described and modeled. As by definition the driving force behind diffusion is a concentration gradient over a boundary layer between two phases. In order to asses the flux of a compound the major variables are the concentration of the compound in both phases and properties over the layer that separates one concentration from the other (Kramer, Van Eijkeren et al. 2007). In modeling it was assumed that diffusion and active uptake are driven only by the free concentration of an agent. Moreover it is assumed that uptake rates are in no way influenced by its bound fractions. However lately there has been some proof of the bound fraction of a compound to have an important influence on

uptake kinetics (Jansen, Blust et al. 2002; Degryse, Smolders et al. 2006; Kramer, Van Eijkeren et al. 2007). The hypothesis for this process was the ability of a ligand to facilitate the transport of a compound from one phase to other. More precisely the concentration gradient in the boundary layer might be altered by this facilitated transport, and as explained above this is the major factor influencing uptake rates (figure 2-1 gives an indication of possible processes).

It is now clear that uptake kinetics can play an important role in toxico-kinetics and that facilitated transport might influence those kinetics. Hence it is possible data *in vivo* and especially *in vitro* target site concentrations over time might be influenced by this phenomenon. This raises an important question namely: When and to what extent can facilitated transport influence data on in vitro experiments and in what way are those influences relevant to in vivo toxicology?

Chapter 2. Theory

2.1 Flux and uptake kinetics

The flux of a compound driven by diffusion into a phase with a lower concentration is described by Fick's first law of diffusion (Equation 2-1 as described by Kramer and van van Eijkeren et al. in 2008 or a different notation equation 2-2 described by Jansen and Blust et al. in 2002 and previously by Van Leeuven in 1999). However, when using this law the assumption is made that diffusion through the boundary layer is the rate-limiting step for the uptake of a compound. The cases in which diffusion is not the rate-limiting step the situation is of no interest to this study. If diffusion is not the rate limiting step then other processes must determine uptake-rates like: intra-phase diffusion, uptake from the uptake surface or association and dissociation rates. The latter point is closely related to facilitated transport, why this point partly determines the relevance of facilitated transport for uptake-kinetics will be explained in section 2-2. The first point seems imminent since if intra-layer diffusion is rate-limiting, enhanced diffusion rate will not influence uptake kinetics (Kramer, Van Eijkeren et al. 2007). The same goes for the second point that is explained by equation 2-3 (Van Leeuwen 1999). Equation 2-1 and 2-2 describe the Flux of a compound, as a function of a concentration gradient over a certain distance.

Equation 2-1: Fick's first law of diffusion (Kramer, Van Eijkeren et al. 2007)

$$J_{r,t} = -D\frac{\partial C_{r,t}}{\partial r}$$

Equation 2-2: Fick's first law of diffusion (Van Leeuwen 1999; Jansen, Blust et al. 2002) The M in this equation stand for the free metal ion and ∂_M means the diffusion layer thickness for a particular ion.

$$J_{dif} = \frac{D_M}{\partial_M}(C_M{}^* - C_M{}^0)$$

In these equations J is the flux of a compound into another phase in mass unit per area unit per time unit (e.g. mol·dm^{-2}·h^{-1}), D is the diffusion coefficient (area unit^2 per time unit) C is concentration at distance from the uptake surface r and time t or in equation 2-2 $C_M{}^*$ is the bulk metal ion concentration and $C_M{}^0$ the concentration at the uptake surface. This function described an uptake rate that is highest at the initial time and increases with a decreasing boundary layer.

The actual term for bio-uptake as explained by Van Leeuwen et al. in 1999 is shown in equation 2-3 but as said above is assumed not to be the rate limiting step so does not have an influence on the uptake flux for situation in the present report. However the formula shows that uptake is only dependent on the concentration at the uptake surface. The latter goes for all uptake processes.

Equation 2-3: Term for bio-uptake form the uptake surface into a phase; C^0 is the concentration of an agent at the uptake surface. J_u^* is the limiting value for uptake and can be seen as a V_{max} in Michaelis Menten kinetics.

$$J_u = J_u^* \cdot \frac{C^0}{K + C^0}$$

If additional uptake processes take place like active uptake by ion channels or trans membrane transporter enzymes, an extra term is often added to the equation also following Michaelis Menten kinetics. (Van Leeuwen 1999; Jansen, Blust et al. 2002; Jansen, Arts et al. 2005; Kramer, Van Eijkeren et al. 2007) The essence of the flux stays the same, an amount of a substance enters a phase over a certain area over time.

2.2 Facilitated transport

Facilitated transport is a process that occurs in most organisms. It involves binding of a substance to one or more ligands in order to facilitate the transport of the substance into a different phase, for example a cell-membrane (Barta, Sideman et al. 2000; Degryse, Smolders et al. 2006; Kramer, Van Eijkeren et al. 2007). In this study the focus will be on facilitated transport as a process that enhances the flux of a substance into a particular phase in a passive way. There are studies that claim to have proof of facilitated transport, by for example Bovine serum albumin for fatty acids, to enhance active transport over cell membranes. In this case the cell membrane would have high affinity receptors for the ligand that binds the substance that is to be transported by active transporter proteins in close range (Barta, Sideman et al. 2000; Marra, Girón et al. 2002). However the latter point is of great interest to natural occurring processes for which the high affinity ligand receptor and active transporter have some connection, any influence of those systems to enhance the potency of toxic environmental agents seems unlikely. Furthermore for biochemists facilitated transport can be defined as the transport of agents by membrane channels or transporter proteins in cell membranes (Berg, Tymoczko et al. 2002; Kitamura, Maeda et al. 2008). This is however also out of the scope of this report. Therefore in this study facilitated transport will be discussed only as a process enhancing the flux of a compound into another phase and for which the driving force behind the flux is diffusion.

The definition for facilitated transport that will be used in this study is: A process that enhances the flux of a compound from one phase to the other without chemically modifying the compound and the driving force behind the flux must be diffusion. The last point is added since this report will focus on the alterations in the concentration gradient in a boundary or diffusion layer caused by an extra flux up to the uptake surface of compound bound to the ligand, and not past the uptake surface.

As a result of the definition given above facilitated transport can never alter equilibrium constants. This means that equilibrium conditions in essence remain the same. If equilibrium between phase has been set the ratio between the concentrations in all phases and the amount of compound bound to ligands should always be the same. However environmental conditions like pH, salt concentration, ligand concentrations and

temperature can alter those condition to a large extent, especially the equilibrium constants between the freely dissolve concentration of an agent and it's bound form (Marra, Girón et al. 2002).

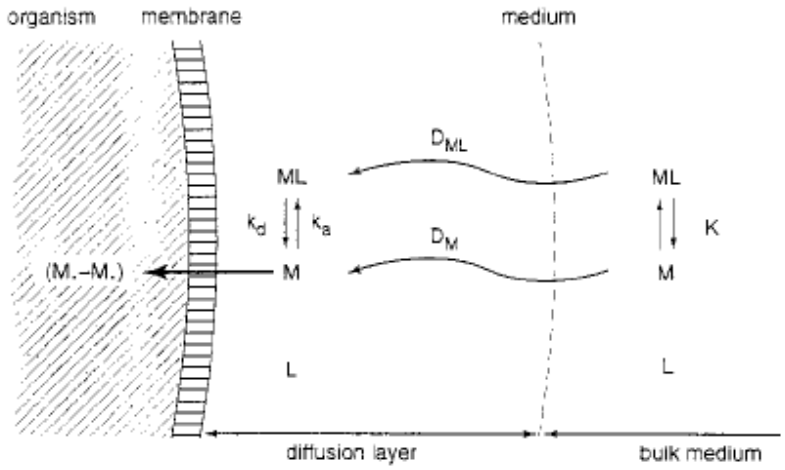

figure 2-1: Schematic representation of facilitated transport of metal ions by ligands. (Van Leeuwen 1999) K stands for the partition coefficients between the free metal ions and the ion bound to the ligands in the bulk medium. D_{ML} and D_L stand for the diffusion coefficient of the bound ions and free ions respectively. The k_a and k_d are the association and dissociation rates of the metal with it's ligands.

Picture 2-1 gives a clear idea of how facilitated transport as defined in this study can influence uptake rates of compounds into another face, in this case a cell. The situation in the picture can easily be translated to other system with a different phase or a different ligand (e.g. passive samplers and proteins (Kramer, Van Eijkeren et al. 2007)).When the information from picture 2-1 is combined with equation 2-2 for the uptake of a compound into a cell the relevance of the theory becomes clear. Firstly by adding a binding matrix to the bulk medium the bulk free concentration of a compound (C_M), as explained by equations 2-3a,b, is decreased (for explanation the example of metal ions and ligands is used (2-3a) but the same equations can be used for protein binding 2-3b).

Equation 2-4: Bulk concentration terms A; Free ion complexation equilibrium term, $K_{m,l}$ stands for the stability constant otherwise known as the partition coefficient. **B; term for protein binding in equilibrium**; C_{bound} is the concentration of an agent bound to protein and K_{prot} the protein binding partition coefficient, C_{prot} is the bulk concentration of the protein.

A;

$$C_M = \frac{C_{ML}}{K_{M,L} \cdot C_L}$$

B;

$$C_{free} = \frac{C_{bound}}{K_{prot} \cdot C_{prot}}$$

The above equilibrium condition are governed by an association and a dissociation rate of the complexes, as displayed by equation 2-5 for metal complexes.

Equation 2-5: Reaction of free metal ions and it's ligands to form complexes; k_a and k_d are the association and dissociation rate respectively.

$$M + L \underset{k_d}{\overset{k_a}{\rightleftharpoons}} ML$$

If in any system the above explained situation would be the only process playing a role only the bulk free concentration of any compound would be changed and therefore the concentration of the compound at the uptake surface would change accordingly. The flux of the compound into the other face would change linearly with the change in free concentration since the diffusion coefficient and diffusion layer thickness are independent of bulk free concentrations (equation 2-1). If however an experimental value for the uptake flux is measured that does not follow a linear relationship with bulk free concentrations after binding to a ligand there is reason to believe that facilitated transport plays a role, this phenomenon has been reported. (Van Leeuwen 1999; Jansen, Blust et al. 2002; Marra, Girón et al. 2002; Degryse, Smolders et al. 2006; Degryse, Smolders et al. 2006; Kramer, Van Eijkeren et al. 2007)

The most important factor determining whether any complex forming might increase the bio-uptake of a compound, apart from the rate of uptake from the uptake-surface, is the relationship between the association and dissociation rates of a complex and the rate of diffusion (Davison 1978; Jansen, Van Leeuwen et al. 1998; Van Leeuwen 1999). To explain this more clearly a new term called *the effective diffusion time* should be introduced. The effective diffusion time is defined as $\partial r^2/D$ in which ∂r is the thickness of the diffusion layer and D is the corresponding diffusion coefficient. This leaves a term with time as unit, and can actually be seen as the time a molecule or complex takes to diffuse from the bulk medium to the uptake surface.

The effective diffusion time can be used to relatively quantify the speed of the diffusion process of a complex in comparison to the free compound. This point is illustrated by The Davison criterium and is extensively explained by Van Leeuwen et al. in 1999 and introduced by Davison in 1978. They show that if the criterium (equation 2-6) is met, the uptake-flux of a compound is driven by both the diffusion of the free metal ions and the complexed metal ions.

Equation 2-6: Davison criterium (Davison 1978)

$$\frac{k_d \delta}{\sqrt{k_a} \cdot \sqrt{D_M}} \gg 1$$

The above criterium is the mathematical way of saying that facilitated transport as defined in this study only has an influence on the uptake-flux if the diffusion of the complexed agent and the dissociation of the complex at the uptake surface are faster than the diffusion of the free agent itself. It can be assumed that this criterium also goes for

compounds other than metal ions bound to proteins or other extracellular matrix carriers. Important to realise is that simply because of their larger size most complexes diffuse slower than the corresponding free agents (Van Leeuwen 1999).

2.3 Ligand binding *in vitro* and *in vivo*

The above explained theory assumes binding of free agent to some ligand in the bulk medium. The bulk medium in this case can be anything from blood plasma and cell culture medium to surface water. Of course facilitated transport can only take place if agents are indeed bound to a large extent to ligands in those mediums. Therefore it is interesting to illustrate some environments both *in vitro* and *in vivo* in which this plays an important role for uptake kinetics.

There are many published examples of *in vivo* metal uptake that does not follow the FIAM model. It has been shown in the presence of organic ligand metal uptake in fish or other organism is higher than expected from the FIAM model. For example the uptake of zinc, copper and cadmium by fish, mollusks and plant is enhanced by dissolved organic ligands in fresh water (Jansen, Van Leeuwen et al. 1998; Jansen, Blust et al. 2002; Meylan, Behra et al. 2004; Degryse, Smolders et al. 2006; Degryse, Smolders et al. 2006; Zhang, Sun et al. 2007). *In vitro* the FIAM model normally describes uptake data for metals better (Meylan, Behra et al. 2004), since appropriate organic ligands are normally not present in cell culture medium or other *in vitro* test systems. This issue should be taken in consideration when extrapolating data from in *vitro* to *in vivo* situations.

There is some published data on enhanced uptake of chemicals due to the presence of BSA in *in vitro* test systems but this hasn't been acknowledge to a large extent yet. (Gülden, Mörchel et al. 2002; Marra, Girón et al. 2002; DelRaso, Foy et al. 2003; Foy and Frazier 2003; Kramer, Van Eijkeren et al. 2007). It is however widely known that BSA and other plasma protein have a great influence on pharma- and toxicokinetics. However the theory of these protein to enhance uptake fluxes in the same way and according to the same physical laws as metal complexes is a new hypothesis. Whether this is of great influence in fluxes from blood plasma to cells is doubtful. *In vitro* the process should definitely be taken into account.

There is also published data on enhanced uptake fluxes of cadmium in Carp in the presence of nanoparticals (Zhang, Sun et al. 2007). Whether this should be considered facilitated transport in the context of this study is discussable. However, the concept is relatively new and shows that there are still many issues concerning uptake kinetics. It also shows that when extrapolating data from *in vitro* situation the lack of millions of compounds that are present in the environment and the relative complexity of real life situations can still influence extrapolation.

2.4 Implications for toxic potencies of chemical agents

From the aforementioned literature examples and theoretical background to facilitated transport, it is clear that bulk ligand concentrations can have a clear effect on uptake kinetics of agents into cells, organisms or other phases. This however is of no real interest if the enhanced uptake itself does not have any influence on pharmacological or toxicological effects. Since equilibrium conditions remain the same in principle the concentration of the agent to which cells or organisms are exposed remains the same with some exceptions.

Facilitated transport can have a great influence on kinetics in toxicological assays, especially assays in which an agent is removed from the intracellular environment by for example metabolism. Since concentrations of the agent do not reach equilibrium conditions because they are removed from the intracellular environment there is a constant flux of compound into the cell. If the uptake is the rate limiting step for metabolism the metabolism rate might be increased. This is supported by studies on metabolism. (Foy and Frazier 2003; Baker and Parton 2007; Nichols, Fitzsimmons et al. 2007) The result of this higher metabolism rate might be a higher concentration of the metabolite in the cell due to cumulative uptake rates or a higher secretion rate from the entire system due to faster metabolism. Baker and Parton in 2007 reviewed that hepatic clearance *in vivo* is often under-predicted from *in vitro* tests. Moreover the under-prediction decreased with higher plasma protein levels *in vitro*.
If the metabolite is actually active this has an influence on the effect of the exposure to the agent. Also if an bio-active agent is removed from a system faster due to higher metabolism rates this may result in a lower effect since the total exposure over time is lower. It is known that the shorter exposure results in a lower effect (Reinert, Giddings et al. 2002).

In other cases an agent might be removed from the extracellular environment faster than the time to reach equilibrium between the system and the cells. As a rule the first step in development of toxicity is always a "battle" between elimination, distribution away from the target site, excretion and detoxication on one hand and absorption, distribution towards the target reabsorption and toxication on the other hand. Facilitated transport can alter the kinetics involved with distribution and absorption processes. It is accepted that plasma proteins only have a effect that lowers toxicity (by binding), by facilitated transport this effect is lower then expected or can even increase the uptake (Kwon, Rutishauser et al. 2009). In this case a higher uptake rate may result in a higher peak concentration in the cells. In this case cells are actually exposed to a higher concentration of an agent. Depending on the toxicologic mechanism it can have a great influence on the actual effect. It is also possible that some agent might reach an effective concentration faster, in other words toxic compound or drugs might start working faster if the uptake rate in the cells in higher.

The last point is a point only applicable to *in vitro* assays. If cells are normally exposed to an agent at a certain dose it normally takes a certain amount of time for the cells to reach equilibrium with the medium. In figure 2-2 a representation of the intracellular concentration of spiked cells in an *in vitro* assay is displayed. For many toxicants the

toxic effect is determined by the dose and the time to which cells are exposed to this dose. If equilibrium is reached faster the actual dose will be the same but the time in which cells are exposed to this dose will be reached faster. Because of this effect the Area under curve (AUC) will be larger when uptake fluxes are increased, therefore the toxic effect will be larger.

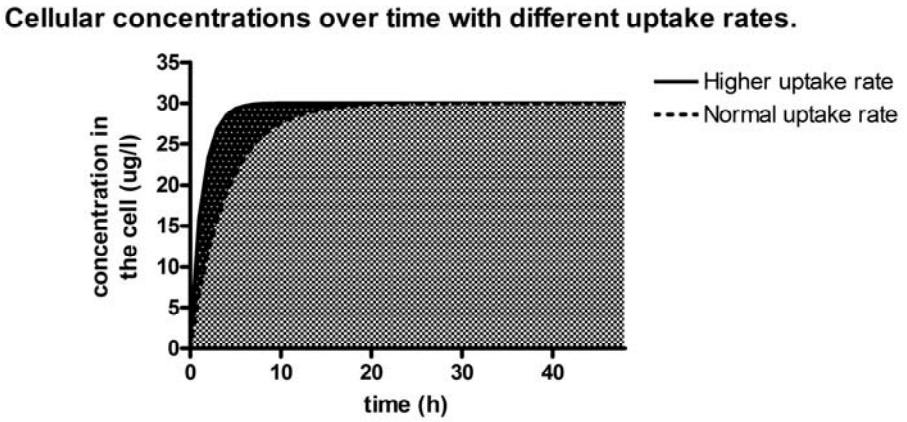

figure 2-2: Example of concentration over time in a cell assay; The dotted line describes the concentration in a cell over 48 hours after exposing the cell to medium with a certain concentration of an agent. The darker area is the increased amount of agent to which the cell is exposed when the uptake rate is higher due to for example facilitated transport.

Conclusion

The question raised in the introduction of this report name: "When and to what extent can facilitated transport influence data on in vitro experiments and in what way are those influences relevant to in vivo toxicology?" can be partly answered now. The parts of the question to which no answers have been found give opportunities for further studies.

First of all it has been made clear that facilitated transport as defined in this study (section 2-1) has an influence on uptake kinetics and that the known laws for metal complexes can most likely be extrapolated to other ligand binding processes. The experimental data as well as logical reason make it very likely that psychical laws like the Davison criterium also go for plasma protein binding and other ligands leading to facilitated transport. This directly leaves important options for further studies in kinetics modelling. It would be usefull to obtain data on how psychical properties of compounds and possible ligands can be used to model diffusion constant and dissociation and association rates as well as diffusion layer thickness. In the same way as those properties are now used in QSAR modelling. In this way given psychical properties of a compound and ligands it might assessed whether the Davison critetium is met. Moreover the psychical properties of the compound and it's ligands might indicate whether facilitated transport might add to uptake fluxes.

The second point is the binding of agents to certain ligands. As yet there are already many studies and publications available on the binding of toxicant to ligands. Mostly the rationale for those studies is and was the assessment of free compound concentrations. Models like the FIAM model and other describing dose-response relationships correlated with free in stead of nominal concentrations are results of those studies. The data of those studies is available and the data might better be described using models that take facilitated transport in account. The information obtained from other studies might therefore be used to assess the influence of facilitated transport to a larger extent. In the future it might be worthwhile to study already published data and expand already existing models with models describing facilitated transport. Data from those studies is valuable in two ways. Firstly the actual data might actually lead to better predicting and higher correlation thereby improving the models. Secondly in large studies taken together can give a good idea about systems and substances for which facilitated transport might play an important role, thereby a model describing facilitated transport might be improved.

Finally the process that is described by any model and is possibly influenced by facilitated transport should describe data and processes that are actually influenced by facilitated transport. For example simple acute toxicity tests on cells will hardly be influenced by facilitated transport since the dose response relationship in those test is not influenced. Facilitated transport only has influence on data that describe or are indirectly describing uptake kinetics. This point is explained in section 2.4, if an agent is removed from the target phase or from the donor phase and equilibrium is not normally reached between to phases, facilitated transport can increase total fluxes, leading to higher concentrations in target phases or higher metabolism rates. In this case the facilitated transport should be modelled and for extrapolation the ligand should be included or removed from *in vitro* assays when possible. Issues with constant dosing assays might be explained to some extent since facilitated transport increases equilibrium rates between two phases thereby makes the dose “more”constant.

It can be concluded that for facilitated transport to be influential certain criteria should be met.

- An agent has to bind to ligands present in bulk mediums.
- For those agents and ligands the Davison criterium should be met.
- The *in vivo* processes to which *in vitro* data apply should be influenced by facilitated transport. The ligands should be absent or prevalent at different concentration in corresponding experiments, or the entire system should add kinetic factor to either the *in vitro* or *in vivo* situation that are normally absent.

If all the above criteria are met it is likely that models in which facilitated transport in included describe actual *in vivo* data better then traditional models.

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