

Letter to the Editor

Reactivity assays: dealing with substances and samples in an *in vitro* world – the solution and the real problem

Dear Sir,

Pease et al. have commented (1) on our letter to the editor in Issue 61 of *Contact Dermatitis* (2). We agree with much of what they say, but it is useful, in the interests of ultimately developing optimal strategies for *in chemico* approaches to predicting skin sensitization potential, to discuss some of the issues further.

Ratio of Reactants in Reactivity Assays

In our letter, we said “A key decision when considering doing a reactivity assay is whether it is the pure substance that is of interest or whether it is a particular sample of the substance, for example a raw material for use in a formulation. If it's the *sample* you're interested in and you want to determine if it has the capacity to sensitize, even if the sensitization may be due to an impurity, then you should do a reactivity assay with an excess of the sample – so even if the main compound is unreactive, the reactive impurity will reveal itself by a significant depletion of the nucleophile. In contrast, if it's the *pure substance* that is of interest, you should use an excess of nucleophile, so that any reactive minor impurities that happen to be in your particular sample don't cause confusion.”

Pease et al. argue that if the assay is coupled with a post-reaction analysis of the reaction products, use of a high test compound to nucleophile ratio is appropriate to address both the impurity and the main component questions, based on the reasoning that products derived from the main component and from impurities can be separately identified and distinguished. Unfortunately, although this reasoning may apply in many cases, it is not universally applicable. If an assay with a large excess of test compound reveals no significant reaction, or if the only reaction products detected are clearly not derived from the main compound, this is an unambiguous indication that the test compound is unreactive under the assay conditions. However, observation of a reaction coupled with identification of a reaction product related to the test compound is not necessarily a correct indication of the reactivity

of the test compound. This is largely because many compounds capable of sensitization are of general structure R–X, R being the structural element that becomes covalently bound to the nucleophile and X acting as a leaving group. Compounds with different X but identical R groups will, if they react, all give reaction products Nu–R. For example, suppose that we have a sample, nominally >97% pure, of 2,4-dinitro-(trichloromethyl)benzene, $\text{CCl}_3\text{C}_6\text{H}_3(\text{NO}_2)_2$. Our chemistry insights suggest to us the possibility, but not the certainty, that this compound might act as an $\text{S}_{\text{N}}\text{Ar}$ electrophile, analogous to 2,4-dinitrochlorobenzene (DNCB), the trichloromethyl anion acting as the leaving group. A cysteine peptide reactivity assay with the test material in 100:1 excess, in which we observe significant peptide depletion, or even measure a rate constant, and in which we identify the reaction product Peptide-S- $\text{C}_6\text{H}_3(\text{NO}_2)_2$, would not show whether $\text{CCl}_3\text{C}_6\text{H}_3(\text{NO}_2)_2$ is reactive or whether a minor impurity (e.g. DNCB) is responsible.

Extent of Promiscuity of Binding to Different Peptide-based Nucleophiles

Pease et al. mention the ‘extent of promiscuity of binding to different peptide-based nucleophiles (e.g. that could in principle lead to greater chances of antigen formation)’ as a parameter that could usefully be determined in peptide assays. This concept merits some further discussion.

In the context of chemical reactivity, promiscuity is the converse of selectivity, the latter being a long- and well-recognized term in chemistry. Selectivity may be defined in terms of relative rate constants. In the Swain–Scott equation

$$\log(k_{\text{Nu1}}/k_{\text{Nu2}}) = (n_1 - n_2)s,$$

the parameter s quantifies the electrophile's selectivity towards nucleophiles Nu with different nucleophilicity values, n . Electrophiles with low s values may be considered promiscuous. Rate constants from full kinetic studies can be used to

quantify selectivity/promiscuity, but it is a well-established common practice, particularly with highly reactive compounds, to use competition experiments in which two or more reactants in excess compete to react with the compound being studied. In the present context, this approach would involve treating the test compound with a mixture of nucleophilic peptides, each present in excess over the test compound, and analysing the final reaction mixture to determine how much of each peptide has reacted with the test compound.

It is important not to confuse promiscuity with high reactivity. This is easy to do when using peptide assays with the test chemical in excess. Electrophile A may give close to 100% depletion of the more reactive peptides and less, but significant, depletion of the less reactive peptides, while electrophile B gives significant, maybe even 100%, depletion with the most reactive peptide, but no significant depletion with other peptides. This cannot be taken to mean that electrophile B is less promiscuous than electrophile A. It simply tells us that electrophile A is more highly reactive, capable of reacting with most nucleophilic groups encountered in peptides under the assay conditions, whereas electrophile B is less reactive, such that under the assay conditions, it gives significant depletion only with the most reactive peptide. It is however safe to assume, for electrophiles reacting by the same mechanism, that the more peptide types a compound reacts with, the more reactive it is.

To our knowledge, there are currently no published studies in the contact dermatitis literature in which promiscuity/selectivity of sensitizers, as distinct from degree of reactivity, has been determined, and there is no evidence that promiscuity/selectivity is a relevant parameter in determining sensitization potency. Unless and until a need arises to invoke this concept to rationalize sensitization data that cannot be explained more simply in terms of reactivity, it seems to us that it is more efficient to adopt the working assumption that it is not relevant. Nevertheless, we accept that this view may not be shared universally. We would recommend, therefore, that if

promiscuity/selectivity data are to be generated, the methodology should be able to distinguish unambiguously between promiscuity/selectivity and the extent of reactivity. This could involve performing reaction kinetics for a selection of peptide-based nucleophiles or competition experiments with the nucleophiles in excess. As a numerical parameter quantifying promiscuity/selectivity, we recommend determining either the Swain–Scott s value, or a variant of s based on a peptide nucleophile scale.

Conclusion

Except for the most basic aspects of hazard identification toxicology, we strongly support the views expressed by Pease et al. regarding the importance of understanding the reaction chemistry relevant to skin sensitization and the importance of deriving universal reactivity parameters in the form of rate constants or their equivalents rather than simply trying to use assay-dependent depletion values. We also hope that the arguments presented in this work will be helpful in the drive to provide the best chemistry input to non-animal predictive methods for skin sensitization.

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References

1. Pease C, Thain E, Zazzaroni R. Reactivity assays – dealing with substances and samples in an *in vitro* world – the solution and the real problem. *Contact Dermatitis* 2010; 62: 256–257.
2. Roberts D W, Basketter D A. Reactivity assays, substances, samples and *in vitro* alternatives: why should we care? *Contact Dermatitis* 2009; 61: 310–311.