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Dear Dr. Stokes,

This public comment is delivered in response to the Federal Register Notice Volume 74, Number 60, pages 14556 – 14557. It addresses the draft ICCVAM BRD on the Hen's Egg Test on the Chorio-Allantois Membrane (HET-CAM) (March/April 2009) with the current Status of In Vitro Test Methods for Identifying Low End Irritancy.
(http://iccvam.niehs.nih.gov/methods/ocutox/PeerPanel09.htm)

Introduction: In the Preface ICCVAM experts remarked on the lines 478 ff that the hen's egg test on the chorioallantoic membrane (HET-CAM) in a previous evaluation did not perform sufficiently to identify severe (irreversible) ocular irritants/corrosives using the EPA, United Nation Globally Harmonized System of Classification and Labeling Chemicals (GHS), and the European Union regulatory hazard classification system. This is in line with the findings of the German validation study (Spielmann et al. 1996, **24**, 741-858, Kalweit et al. 1990) and was the reason why the German outcome of the validation exercise proposed to use a combination of two methodologies to identify severe hazards more reliably. But proving such approach was not in the focus of the ICCVAM program.

ICCVAM now is reviewing the validation status of the HET-CAM for the identification of non-severe ocular irritants (that is, those that induce reversible ocular damage) and non-irritants. The Ocular Toxicity Working Group (OTWG) of ICCVAM and NICEATM has prepared a draft background review document (BRD) that summarizes the current status of this test methodology based on published and other submitted information.

General remarks: In its *Executive Summary* the OTWG experts have summarized that the CAM has been proposed as a model for a living membrane, since it comprises a functional vasculature, which does mean that the structural tissue damage induced by irritant chemicals can best be observed by the beginning of **vascular leakages (bleeding)** (line 575ff). A second additional information of structural damage induced by irritant chemicals can be the **coagulation** of structural and functional tissue components like proteins und carbohydrates (e.g. after protein denaturation, i.e. loss of functionality and solubility (which must not be irreversible per se). "**Coagulation**" is not equal to "**protein denaturation**". It can be the result of structural impairment (denaturation) of physiologically relevant gels accompanied by the loss of solubilisation and subsequent precipitation of structural constituents. This process can lead to cloudiness and/or opacity of originally clear and transparent gels playing obviously an important role in the visual process in the cornea.

Both processes tissue and cellular damage (bleeding) and coagulation ((cloudiness/opacity) play a role in the ocular tissues, the conjunctivae as well as in the corneal tissue. Coagulation as characteristic part of the corneal opacity can easily be observed and play a major role in estimating the impact, i.e. the severity and duration of especially strong irritants in the Draize scoring system. Coagulation does not reflect all types of corneal damage per se, and vice versa the damage of cellular matrices in the cornea ("area of depth and injury")

must not be accompanied by coagulation and consequently lead to opacity, these are two different qualities of damaging effects in the tissue. As a result there are two endpoints: i) vascular lyses, hemorrhages and bleeding that becomes visible, and ii) physicochemical damage and perturbation of transparent physiological gel matrices that become cloudy and opaque.

The confusion of terminologies that appears to still exist not only in the executive summary of this BRD and therefore might have influenced the outcome of this analysis is also characteristic for some older HET-CAM protocols, and in particular for the oldest version proposed by Lüpke et al.. There exist a number of protocols and modifications thereof that partly uses additional endpoints like **hyperemia** and/or **vascular lyses** that cannot be clearly identified or differentiated without using special microscopic equipment. But often enough this was not verified in the protocols. In our experience vascular lyses was not considered to be a valid separate endpoint but the prerequisite of the easily observed bleeding. At a later state vascular structures can disappear (in particular if certain types of surfactants have been applied). Similar observations showed that hyperemia cannot be differentiate without stereo microscope from slight diffuse bleeding. But hyperemia when it really occurs (mostly after treatment with slightly to non-irritant chemicals with particular properties) can be depending on the dose and time reversible phenomena of the capillary vasculature of the chorioallantois tissue.

Therefore it is not surprising that out of the large number of cited papers and procedures only few data sets seem to allow a comparison and subsequent biometrical analysis. As a result of this consideration there seem to be need to put together hemorrhages and vascular lyses for biometrical analysis and better leave out hyperemia for data analysis.

Validation Data Base (Line 587ff): The definition of in particular chemical classes more than product classes is a complex task. Accordingly the table in **Appendix A** is not very consistent. Since the biometrical analysis has been performed according to chemical or product classes it is may have an impact on the results. Some out of many examples might be given for illustration:

- **Anisole** is put into the classes; Ether and phenol, but
- **Phenol** itself is classified as alcohol, therefore it is not clear whether phenols are considered as alcohol.
- **Glycerin** (CASRN 56-81-5) is taken separately although it is a (German) synonym of
- **Glycerol** having the same CASRN. (*Compare also n-Butanol and Butanol*)
- **Potato Starch** is put into the class of “Hydrocarbons” although it belongs to the non-irritant Carbohydrates.
- **Potassium Laurate** seem to be the potassium **salt** of a fatty acid or carboxylic acid, but not a cationic surfactant, there are a lot of
- Inorganic and organic salts among the chemicals that are summarized as carboxylic acids although they might act as an anion, which is an essential difference.

Just to mention some aspects of classifying chemicals. The inorganic acids are not mentioned as such. This is of interest because strong acids organic and inorganic as well as alkalines must not be tested in vivo! - according to the OECD TG 405. A number of salts are classified as surfactants, may be because they act as such, but chemically they are organic salts like: Benzalkonium chloride and Sodium Dodecyl Sulfate.

This issue may hold true also for the other BRDs not reviewed in this paper. This list needs to be reviewed very critical for refining the results.

It seems to be more important from the viewpoint of applicability to sort the materials according to solubility in watery systems or in oil phases, as already done for the large document published in 1996 by Spielmann et al. in ATLA and several preceding papers, e.g. Kalweit et al. 1990, which contain all relevant parameters of the SOP which are missing in

the Appendix B1 and which might comprise the largest set of consistent data in this background review document.

This leads to the last remarks for the use of animals (BRD line 1900ff): In Appendix B1 it remains unclear how the days of embryonic development are counted. The process used to start after collecting the eggs, mostly with the artificial fertilization, and shipment to the laboratory, where then the start of the breeding is defined in a narrow slot before starting the breeding. Relevant are then the nine 24h-periods of breeding and development prior to testing in order to avoid the progress in the development of sensory nerve fibers.

The remarks collected and presented here comprise a brief summery and due to the time constrains for public comments not all possible and necessary comments.

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