Comments on the HET-CAM part of the Expert Panel Report of March 2005 and on the adapted "Protocol for the HEN'S EGG TEST on the CHORIOALLANTOIC MEMBRANE" (Appendix A)

Provided by the Members of the COLIPA/SCAAT Eye Irritation Task Force:
Wolfgang Pape (Beiersdorf, Hamburg, Germany)
Claudine Faller (Cosmital Wella, Marly, Switzerland)
Béatrice Le Varlet (LVMH Recherche, Saint Jean de Braye, France)

Comments on the HET-CAM part of the Expert Panel Report of March 2005

1. *Executive Summary*

1.1 *Page xi, sentence: 'It was proposed…be re-tested in a modified HET-CAM (e.g. using a lower test concentration of the test substance) to confirm the results."*
The wording '*modified'* could be misunderstood, in the sense of using a different protocol etc., where it is obviously meant to test the substance at lower concentrations in the sense of looking for dose responsiveness. In order to reduce the number of false-positives in the INVITTOX 1992 protocol (mentioned later) it was proposed to use 10 % test concentration where possible. Interesting enough similar thoughts may be valid also for the Rabbit eye irritation test OECD TG 405, if certain physico-chemical properties of a test substance might influence the bioavailability. These effects need in particular to be considered if so-called in vitro data are compared with in vivo Rabbit Eye data.

1.2 *last paragraph: 'The Panel further recommended that an evaluation be conducted to determine the relationship…severe irritants'.*
The meaning of this paragraph becomes not really clear, because the HET-CAM protocol covers only very early acute tissue injuries like setting a wound, whereas the long-term effect in rabbits or humans is dominated by following inflammatory processes and those of wound healing. Already the comparison of HET-CAM data determined within 5 minutes with rabbit data taken 24 h after instillation might be a mistake in view of the role of inflammatory responses influencing the reading of the scores. We should not forget that in animals the inflammatory immune response is used to assess the severity of the tissue damage, i.e. necrosis of cells, structural changes of functional and structural proteins, polysaccharides etc.. We have to learn and think about what happens at molecular level and how does this relate to the immune response used for estimations till now.

2. IV. *HEN'S EGG TEST on the CHORIOALLANTOIC MEMBRANE (HET-CAM) should be the correct title, as a short term in ovo organ/cell test.*

2.1 *1.1.1 the last four bullet points:*
The day 9 or 10 (depends on counting method starting with 0 or 1) taken for testing was due to the fact to avoid that the CAM was not completely developed and closed. On day 7 it may happen that the test substance after application disappeared within the egg and did not stay on the membrane, i.e. it will react elsewhere within the egg.

2.2 *1.1.3/4 etc. Similarities and Differences…, Mechanisms…*
It reads as if the exposure of the conjunctiva to a chemical directly results in an immunolgical reaction. But this is not the case. The first and maybe slightly slower reaction within the conjunctiva is tissue and cellular damage (setting the wound) which then results in the immune response: named inflammation. And it seems already to be assumed concerning the rabbit test that there is a direct relation between severity of the tissue injury and the inflammatory response, which might allow to conclude that the severity of the inflammatory response maybe a direct measure of the irritancy of the substance, which primarily means, e.g. necrosis of tissue cells and more or less dramatic changes of other structural entities.
Since all in vitro and ex vivo tests lack of "inflammatory response" we have to consider this aspect very carefully and should not continue to mix up causality and response. In our early experiment about 20 years ago we therefore used scores determined 1 h after administration of test articles.
Therefore and as a matter of fact '*mechanistic similarities'* can only be considered at cellular and molecular level and this has to be understood and always kept in mind.
The pragmatic rabbit eye approach which seems to help us to predict hazards for human beings properly deals with uncontrolled and less known mechanisms of tissue damage within the conjunctiva, the cornea and the iris. In particular the conjunctiva respond with inflammation, whereas the cornea presents opacity as follow up of misbalances of tissue homeostasis, as result of protein coagulation and cellular necrosis, depending on the nature of the properties of the test chemical and its concentration.

If terms like mechanism are used, it has to be clarified what they should mean, they must be properly defined. Pathophysiological mechanism versus chemical/biochemical molecular mechanisms.

2.3 *1.3 The Regulatory Rational becomes more complicated and needs new Data Interpretation Procedures!?*

2.4 *4.2 Interpretation of data…*
The scoring of "discharge" needs particular consideration, because strong discharge may result in a lower in vivo response, which cannot be accurately compared with ex vivo and in ovo data (without any discharge). They can be considered as "in vivo false-negatives"!!!

2.5 *4.5 Human ocular data…*
Human data as far as available can only be used for better understanding of rabbit data and corresponding alternative data if there are inconsistencies due to the different endpoints used for estimating irritancies.

2.6 *4.6. Reliability of in vivo data…*
For a reliable interpretation of rabbit in vivo data knowledge of the properties of the test substance (chemical and in particular preparations) are needed and included, because inconsistent animal data may origin from differences in the behaviour of the substance instilled into the eye. As mentioned above, discharge can play an important role, resulting in change concerning the exposure and the scoring. The in vivo data need as thorough or even more thorough controls, if they should be compared with ex vivo and in ovo alternatives.

2.7 Final remark concerning the HET-CAM data evaluation
Instead of using one or the other of the different scoring systems described in the literature, it is recommended to deal with the directly measured "reaction times" determined according to the standard protocol. There is no need, in contrast it may be dangerous to calculate artificial scores for curious classifications. According to the finding of the Spielmann et al. paper (ATLA 24, 1996) severe irritants and corrosives may be identified within a tight reaction time frame or irreversible findings may need longer reaction times if there are no direct irritant but acute toxic effects.

Comments on the adapted "Protocol for the HEN'S EGG TEST on the CHORIOALLANTOIC MEMBRANE" (Appendix A)

1. Purpose and Application

According to paragraph 2, line 23 the accuracy and reliability has not yet been formally evaluated for other classes of ocular irritancy than for corrosives and severe irritants. This does not hold true, since the test is used in the area of low and non irritant substances and formulations with good experience in various cosmetic companies.

3.1 Source of Chicken Eggs

It should be clear, that the eggs have to be fresh, which means depending on the logistics delivered within few days (1-2). Breeding should after appropriate storage (conditions ?...) start within the next few days, which means altogether < 7 d to avoid unnecessary lost of viable eggs.
The eggs should be candled during breeding/hatching to control development and to sort out defective or non-fertilized eggs.

4.0 Test Substance Preparation

If ever possible substances should be tested as for instance as 10 % solution beside testing as such in order to avoid the generation of to many irrelevant data, e.g. testing of 30 and 15% solutions of SDS may lead in the rabbit to questionable data. Why then test this substance in the HET-CAM as 99.9 % solid?
Line 73. Dilutions should be prepared on the same day as testing and best prior to testing.

5.1/2 Negative Controls and solvent controls should be performed to make sure that the solvent used does not affect the chorioallantoic membrane in a way that might influence the test results.

5.3 Positive Controls

What sort of positive controls are made in the Draize eye irritation test to control that it works? If a positive control is performed it can only make sense to control the person's reliability and fitness and to control repeatability and reproducibility.

5.4 Benchmark

In the EC/HO and the COLIPA Validation projects a HET-CAM Protocol was used including a surfactant as benchmark, which fulfilled the conditions mentioned in this section and it was used to calculate ratios between the individual irritation scores and the benchmark in order to better classify the test substances. The benchmark was according to high quality in vivo rabbit data (OECD TG 405, GLP) classified as R 36.

6.1 Treatment Groups

From our experience it is sufficient to perform only one to three control experiments per day and not for every test substance for practicability reasons. Since, if you perform ten or twenty tests per day, then at the end of the day you may have a lot of control data but only few data on your test articles.
The whole procedure should be oriented on practical aspects and not only on formal ones.

6.2 a Prior to the **CAM Preparation** there is need to give some recommendation and information on the hatching/breeding process, because otherwise you will not find a well developed CAM in your eggs.

Sorry but that is misunderstood!!! The breeding should be started as early as possible after fertilization (< 7 days), but the CAM preparation should not be done earlier than 9 or 10 d of breeding on candled eggs with a clearly developed chicken baby and the corresponding CAM.

a. control of the eggs for setting them into the incubator
b. place them on the rotating trays with about 5 automatic rotations per 24 h.
c. Incubation conditions should include a saturated humidity of about 60 % r.h. at a temperature which can vary between 37 and 38 °C.
d. Incubation is stopped the day before testing, which is day on day 8 or 9, eggs are candled and stored in the incubator again setting them with the larger end upwards without rotation and leave them over night to ensure that the air bubble or cell is placed at this broad upper end for testing the next day's morning.
e. Prior to testing candle the egg's air cell for correct opening the egg shell.
   And then continue with point f.

6.4. Observation

It needs to be added, that if the reaction time recorded for a pure concentrated test substance is < 10 sec, it is recommended to redo the test with the diluted substance in an appropriate solvent, e.g. at a concentration of 10 % in a standardized way to get comparable approaches. This process was generally accepted during the German Study, because the recording of very short reactions times may lead to an increase of variability. Such short reactions times mean in general severe irritant or corrosive as result.

7.0 Evaluation of the Test Results

To compare data from different persons or laboratories it is best not to use the "irritation scores", but the individual reaction times for all observed endpoints. Where haemorrhages and coagulation (intra- and extravasal) are the important parameters for severe irritants. Vascular Lysis is not generally seen with each type of chemical class, but with surfactants.

Finally we have to add, that there is no procedure described in the Appendix A protocol that adequately addresses the handling of solid and poorly soluble materials.

******