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May 5, 2005

William Stokes, D.V.M., D.A.C.L.A.M.
Director, NICEATM
National Toxicology Program
P.O. Box 12233, MD EC-17
Research Triangle Park, NC 27709

Dear Dr. Stokes:

This public comment is delivered in response to Federal Register Notice Volume 70, Number 53, Pages 13513-13514. It addresses the Expert Panel Report on the Evaluation of the Current Validation Status of In Vitro Test Methods for Identifying Ocular Corrosives and Severe Irritants", March 21, 2005.

We believe that the BCOP assay provides important and reliable information about the potential for, and the mechanisms of, chemically-induced eye irritation. Such information can be extremely valuable to both industry and the regulatory community.

The focus of these comments will be Chapter III, Bovine Opacity and Permeability Test Method and the associated sections of the Executive Summary. The comments will follow the progression of the report through the 12 sections. Where specific issues are addressed in more than one section, they will be addressed when the issue first appears.

The authors of this section are to be commented on the considerable effort expended in reviewing the BRD, providing their independent analysis, and preparing this extensive report. Their work is particularly important given the original negative conclusion of the BCOP BRD. We agree with most of their report. Therefore, these comments will focus primarily on points of clarification, the addition of technical detail unavailable or perhaps overlooked in the review, and a limited number of points where our experience differs from the conclusions of the panel.

We would also suggest that improvements be made to the process of conducting reviews of new toxicological methods. We agree with the minority opinions of Drs. Freeman, Stephens, and Theran concerning confusion as to the charge to the Expert Panel and the sense that the BCOP subgroup was put under pressure to retract the subgroup's original conclusions. It was clear from subsequent conversations that many audience members felt the same sense of confusion, as well as surprise that the BCOP subgroup's original conclusions were rejected. We propose that the context in which a new method is proposed to be used is stated clearly in the charge to the Panel at the onset, and that the type of conclusions the panel is to draw be made clear. We also suggest that the role of Expert Panels (or similar groups) in the validation process be discussed and agreed to with the Scientific Advisory Committee for Alternative Toxicological Methods.

Sincerely yours,

John W. Harbell, PhD.
Chief Scientific Officer

Rodger D. Curren, Ph.D.
President

21 Firstfield Road
Suite 220
Gaithersburg, MD 20878
tel: 301.947.6523
fax: 301.947.6538
www.iivs.org

Section 1 BCOP Test Method Rationale

It is very helpful to understand something of the mode of action leading to changes in corneal opacity (*in vitro*). The opacity and permeability scores, even without histology, do provide considerable insight into the action of the test article on the cornea. Those materials that act through membrane lysis (e.g., anionic surfactants) induce increased fluorescein passage without appreciable opacity. The same kind of effect is observed by mechanical removal of the epithelium (Harbell, unpublished). Acids, such as trichloroacetic acid, produce coagulation of the proteins and high opacity but little or no increase in fluorescein passage (Curren et al, 2000 and Ubels et al. 2004). Saponification of the cornea leads to appreciable increase in both permeability and opacity (through destruction of the epithelium and denaturation of the stromal collagen)(Curren et al., 2000). Organic solvents produce a combination of membrane lysis (destruction of the epithelial barrier) and denaturation of the proteins and thus an increase in both opacity and fluorescein passage. The data on ethanol provide a good example. Those materials that produce oxidative damage (e.g., peroxides) or direct alkylation of macromolecules, may produce severe damage without producing a corresponding increase in direct opacity or permeability scores *in vitro*. With such materials, histology is essential and this approach was discussed at length in our public comments on the BRD in November. Mustard (HD) is a bi-functional alkylating agent that can cause temporary (low dose vapor exposure) or permanent (high vapor exposure) blindness in humans and has been studied for over 80 years. When sight is recovered, there is often a life-long problem with corneal epithelial ulceration because of the breakdown (digestion) of the basal lamina during the acute phase of the injury (see mention of lesions in section 3.1, final paragraph, page 63). Furthermore, repair capacity in the eye can be reduced through damage to the stem cell population (Friedenwald et al., 1945 and Blodi, 1971). After the initial exposure, there is the onset of opacity (delayed 6-8 hours in both humans and rabbits) accompanied by a breakdown in the epithelium and loss of keratocytes (Maumenee and Scholz (1948) and Petrali et al. (2000)). The changes after mustard exposures are consistent with the observations of Maurer and Jester that histological changes will be observed in the cornea. The BCOP protocol with extended post-exposure incubation period (e.g., overnight) was designed to detect this type of delayed damage and has been used successful for assessing alkylators and oxidizers (see examples in Curren et al., 2000, Gran et al. 2003 [BCOP BRD Appendix H3]).

The BCOP assay focuses on the cornea as the primary tissue of concern in severe eye injury (see Maurer et al. 2002). The BCOP is also not intended to quantitatively assess very mild materials unless extended exposures are employed and histology is used. The concern raised in the BRD suggests that conjunctival injury would occur in the absence of corneal damage. Although this does happen, we are skeptical that such conjunctival injury (without corneal injury) would persist for an extended time period. It should be noted that in the GHS categorization scheme, conjunctival scores are not evaluated for the classification of severe (category 1) except in the area of persistence. Conjunctival injury can be very serious when sensitization has already occurred (e.g., Lash Lure) but the Draize test, performed on naive rabbits, is not a test for sensitization. It is important to maintain the distinction between assays for irritation and those for sensitization. To quantitatively

address the mild to very mild range of irritation potential, the tissue construct models provide the appropriate resolution (Ghassemi et al., 1997).

The inability of in vitro tests for ocular irritation to detect/predict systemic toxicity (section 1.2.1) was raised by the Expert Panel chair in his opening remarks and was included in the BRDs for all four *in vitro* assays. Several points should be considered in evaluating the importance of this "deficiency". First, much of the testing required for regulatory submissions is performed on formulations where the systemic toxicity of the ingredients is known. Second, a 100 mg dose to a 2-3 Kg rabbit is quite high. To be useful in predicting human systemic toxicity, the doses should be rather more realistic. Most important, the proponents of this concern need to show that proper evaluation of systemic toxicity through the oral, dermal, or intravenous routes of exposure could not have predicted the few instances of unexpected, systemic toxicity reported.

The work of Drs. Maurer and Jester (and their collaborators) provides the fundamental basis on which the predictive capacity of early lesions in the cornea can be used to predict both the degree and duration of the irritation response. This essential body of work was largely ignored in all of the BRDs and so it is particularly important that it has been presented in this chapter (sections 1.1.2, 1.1.4, and 1.2.1). This approach would also seem appropriate for evaluating the isolated eye models as well.

Several possible protective mechanisms are discussed in 1.1.4. These include tearing (both for flushing and buffering) and blinking. Blinking and tearing do remove the test material at some rate (perhaps different between rabbits and humans). The rate is not normally measured in the Draize test and probably varies greatly among different types of test materials (physical form, induction of tearing, blink rate, etc). Thus, the in vitro tests do not claim to model the exact exposure (time x concentration x volume) *in vivo* because it is simply not known. We do know that some solid (granular) test articles may be found in the lower conjunctival sac as long as 24 hours after installation (M. Prinsen, personal communication). One of the advantages offered by the *in vitro* assays is the ability to expose the test system for a fixed amount of time at a fixed concentration. In contrast, the effective exposure (volume x concentration x time) cannot really be controlled (or even easily measured) in an in vivo system. Tears can alter the effective exposure by flushing, dilution, and buffering. Buffering of weakly acidic or alkaline materials may be important in accidental exposure but its impact on the Draize test is not well documented. One might well wonder how the 30 µL tear volume will buffer 100 mg or mL of test substance instilled into the eye.

We agree with the panel's conclusion that "a sufficient mechanistic basis for the BCOP test method has been established".

Section 2. Test Method Protocol Components

Section 2.1 raises several technical points that we would like to address in turn.

1) Age of cattle from which the eyes are isolated: The eyes are by-product of cattle slaughtered for human consumption. The conditions in an abattoir are not those expected in a research laboratory nor does the end user have strict control over the cattle that are processed. Several things are helpful in obtaining good eyes (corneas). Not surprisingly, good communication with the abattoir owner/manager is essential so that the goals and requirements of the program are clearly communicated. While the miniscule revenue from the sale of eyes likely would not increase the propensity to slaughter, it is worth some time and effort to reward proper harvesting. Cattle are often processed in groups depending on the intended use of the meat. Thus, the older (less desirable) animals may be avoided. Taking the eyes far upstream in the process reduces the incidence of post mortem damage (scratches, etc). Having a single employee dedicated to removing the eyes is also helpful. No matter how careful the abattoir might be, there will be some fraction of the eyes that have preexisting spots of opacity and small scratches. It is essential that each eye be carefully checked for damage before the cornea is removed and again after the corneas are mounted. It is not unusual to discard 30% of the corneas obtained.

2) Use of antibiotics in the transport medium: The corneas are held (during isolation) and transported in cold Hanks' Balanced Salt Solution containing 100 IU/mL penicillin and 100 µg/mL streptomycin. At 4°C, the use of antibiotics may not be very effective or necessary, although some reference to published literature would be helpful for this analysis.

3) Zoonotic disease: The BCOP assay is used to test a wide range of materials, some with severe eye irritation potential. Thus, proper personal protective equipment is essential even without zoonotic disease concerns. Lab coats, gloves and eye protection should be required at all times. Proper laboratory sanitation and tissue disposal are also expected.

4) Culture Medium: Minimal Essential Medium (MEM) with 1% fetal bovine serum was the culture medium designed by Drs. Gautheron and Sina. It continues to be the standard. The addition of 1% serum was intended to provide some protein to the medium. It is, by far, the most expensive component of the medium. As we are not expecting that it provide the kind of "growth enhancing components" required in cell culture systems, we might well substitute Newborn Calf Serum at 10% of the cost. It may also be possible to simply remove the protein all together. BSS plus is distributed by Alcon Laboratories for ophthalmic use and might be considered in place of MEM. However, MEM costs ~ $17/500mL while BSS plus costs ~$37/500mL.

5) Use of water or saline to dilute test articles: Discussions with laboratories performing the Draize test revealed that in their practice, test materials are often diluted in water when that is the appropriate solvent to model end use exposure. Testing of personal care products (e.g., shampoos) is a good example of this approach (see Gettings et al, 1996). In addition, when appropriate to the study, the rabbit eyes are rinsed with water as that is the "liquid" most readily available in case of an accidental exposure (DiPasquale and Hayes, 2001).

For the BCOP assay performed on personal care and household products (that might be diluted), we routinely use water as the solvent and compare the test article activity to control corneas treated with water. We have compared water and saline and see no difference in opacity, permeability or histology in the controls (Figures 1 and 2, taken from

the same study). The testing of solid phase pharmaceutical intermediates (poorly water soluble powders) is performed as a 20% w/v suspension in either water or saline, as the client wishes. In either case, the goal is to apply the test article by allowing it to settle out of the suspension over the corneal surface (the technique developed by Gautheron and Sina). Depending on the solubility of the test article, some portion of the chemical may go into solution during the 4 hours of incubation. Our preference for water as a diluent is based on the desire to allow solute to form without instantly pushing an isotonic solution (saline) into the hypertonic range. Whether water or saline is used, buffering activity in the medium must be avoided.

Figure 1. Negative control cornea treated for 4 hours with 750 µL of sterile, distilled water

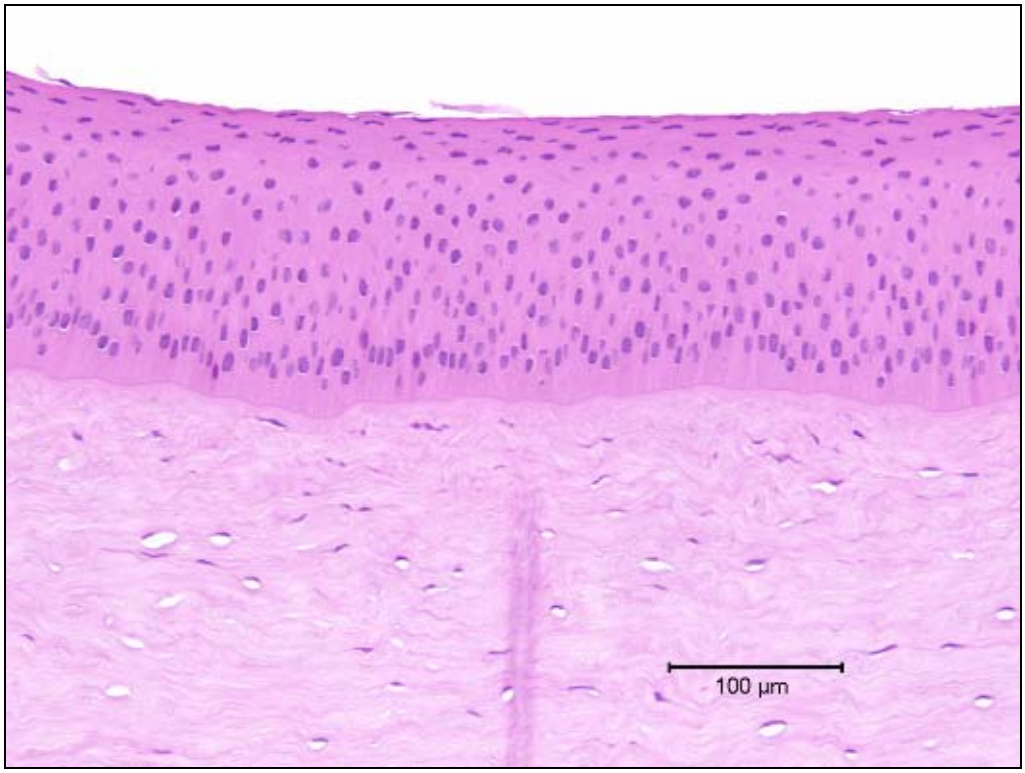
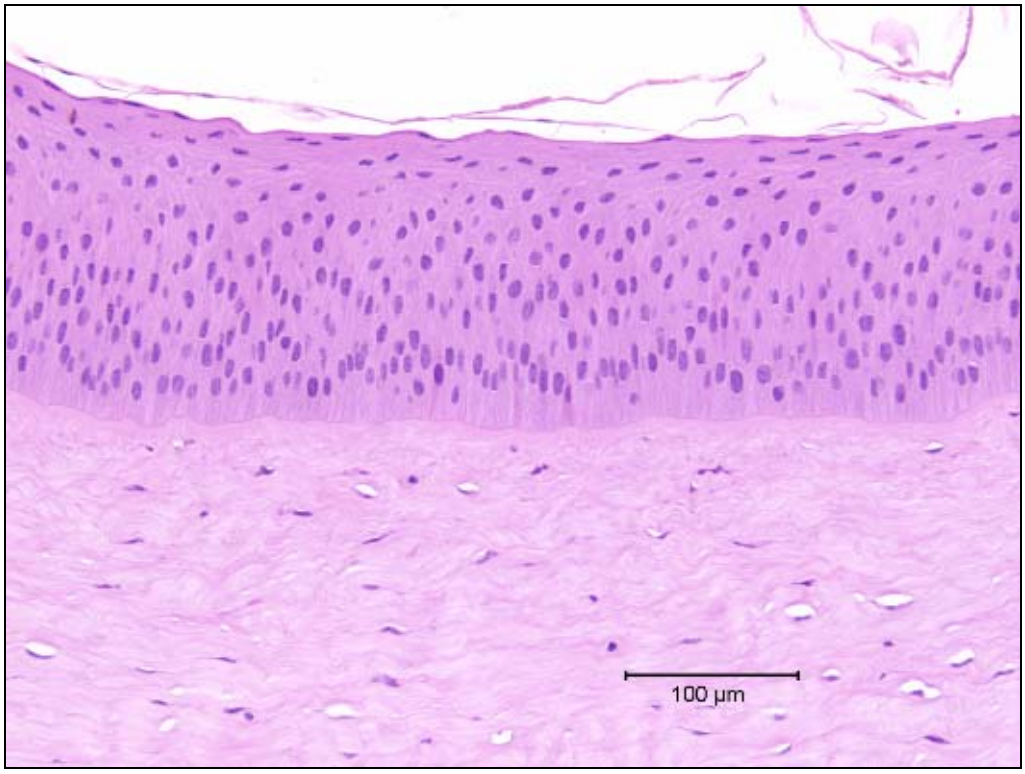


Figure 2. Negative control cornea treated for 4 hours with 750 µL of sterile, distilled 0.9% saline



In section 2.7, the report repeats the concerns about the use of distilled water in a rather more forceful fashion but without supporting documentation to support its concerns. The proposition that distilled water breaks down the epithelial barrier and damages the bovine cornea in vitro is not supported by hundreds of studies in our hands (including histological evaluation of the corneas). The report also calls for the measurement of osmotic activity and cautions that solutions with osmotic activity above 1000 "are known to be damaging to the corneal epithelium" (no reference provided). The report does not suggest how osmotic activity data might be used, or how to relate the results to a Draize test where that measurement is not performed.

6) Cornea holder: All of the BCOP data evaluated in the BRD - except those of Casterton et al. (1996) which used a slightly different holder, but one which also utilized an "O"-ring - were developed using the standard cornea holder from Spectro Designs and Op-Kit opacitometer. This holder is based on the Ussing chamber except that an O-ring, in the posterior half of the chamber, is used to provide focused pressure against the anterior half of the chamber to seal the cornea. The opening that exposes the center of the cornea is 18 mm in diameter and the O-ring is located 1 mm from the edge of the opening (a 20 mm diameter). However, tissue in this 1 mm zone is covered on both sides by the flat surfaces of the holder. Thus, the effective zone for test article exposure, opacity measurement, fluorescein penetration and histological assessment is the 18 mm diameter (2.54 cm^2) central zone. Tissue external to the central zone (O-ring and beyond) is not exposed to the test article and does not participate in the analysis. We use the term "crush zone" in

communicating to the histology laboratory to indicate the excess tissue that may be trimmed from the cornea before embedding to facilitate proper alignment in the paraffin mold.

Figure 3. Photograph of the Spectro Designs holder with a cornea being mounted. The cornea is placed, with the endothelial side down, onto the posterior half of the chamber. The O-ring is under the cornea in this picture. The anterior half of the chamber is very carefully placed onto the posterior chamber so as not to slide the cornea from side to side while aligning the chamber halves.

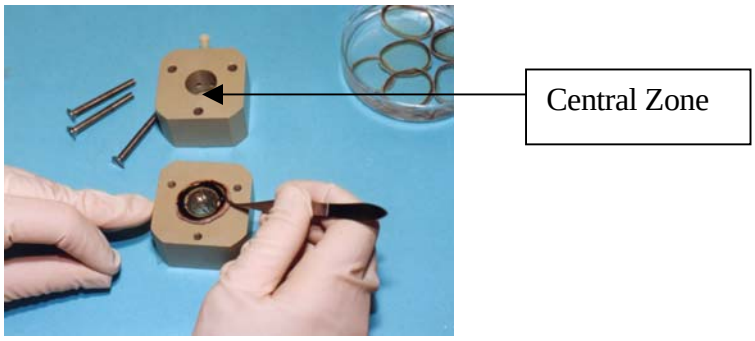
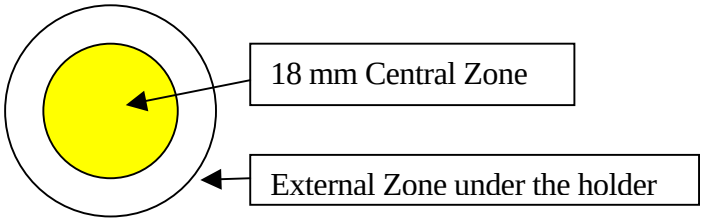


Figure 4. A cartoon, of the mounted cornea, that shows the central zone that is exposed to the test substance and evaluated for changes in opacity, permeability to fluorescein and tissue structure.



We hope that this explanation addresses the concerns expressed in paragraph 6 of page 60.

Casterton et al., 1996 and Ubels et al., 2000 used a modified holder design, produced by Amway Corporation, that was made to fit into a spectrophotometer rather than an opacitometer. This is also the reference holder discussed in the Ubels et al. papers of 2002 and 2004. In Ubels et al., 2004, the authors give the surface area of the central opening as 2.26 cm^2 that would indicate that the central zone in this holder is 17 mm in diameter. As with the Spectro Designs holder, an O-ring is used to focus pressure between the posterior and anterior halves of the chamber (see Figure 1A, Ubels et al., 2002).

In their 2000 paper, Ubels et al., examined the induction of opacity and change in hydration of corneas as a result of short 30- to 60-second exposures with 13 different materials. They compared these results with historical values for a 10-minute exposure from Casterton et al., 1996. Interestingly, the two protocols differ in both the incubation temperature (35ºC for Ubels and 32ºC for Casteron) and length of incubation after exposure (3 hours and 2 hours respectively). They had intended to quantitatively measure endothelial cell damage as a function of test article treatment and did present some comparisons

between the 1-minute and 10-minute exposures. Endothelial cell staining was performed on 8 mm button punched from the corneas. They removed the corneas from the holders to prepare the buttons and before staining the endothelium. In plate 2, they report their observations before and after mounting the isolated corneas in the Amway holders. They show an example where 20% of the endothelial area has stained with trypan blue (indicating endothelial cell damage). These observations were reported as the impetus for the development of the new cornea holder that clamps the cornea on the sclera. This holder is also purported to maintain the normal (in vivo) shape of the bovine cornea.

As the endothelium is just a single layer of cells on the posterior surface of the cornea, it is the cell layer most subject to "artificial" damage in the performance of the BCOP assay. Furthermore, in the histological evaluation of the depth of injury, the loss of endothelial cell integrity would be an important finding in predicting severe irritation potential. Thus, it is to everyone's advantage to retain a functional endothelium on the isolated cornea.

The endothelium may be injured in many ways. One of the more common situations is the over-stretching of the cornea as the accessory tissue layers are peeled away (iris etc). This kind of damage is first seen as fine trypan blue staining in cells along the axis of the stretch (Harbell, unpublished). After a period of time (either mounted or not) the cells begin to separate from the Descemet's Membrane and the trypan blue staining becomes intense (and the alizarin red staining is lost). Air bubbles and other air/liquid interface sheer forces can quickly rupture the cells subjected to these forces (Kim et al., 1997). Mounting of the corneas is also a time of concern. The wet cornea must be carefully laid onto the O-ring and not dragged across it into position. The anterior half of the chamber must be placed directly onto the posterior in its final aligned position. Once the screws are in place, the holder must not be opened to reposition the cornea. Any error in mounting requires that the cornea be discarded. At the end of the assay, the corneas tend to stick to the O-ring and so they must be carefully peeled off in preparation for fixation and histology. It seems reasonable that this peeling operation will damage the endothelium close to the O-ring (probably to various degrees depending how much sticks).

We have now begun to examine freshly isolated corneas and those mounted in the standard Spectro Design holders. Preliminary observations show some loss of endothelial cell integrity in freshly isolated corneas when too much force is applied to remove the accessory tissues. However, properly isolated corneas show only occasional focal lesions. After mounting and incubating for 3 hours, the corneas were stained, in place, as they would be during the assay. Figure 5 shows the edge zone with some trypan blue staining as expected. Figure 6 shows a zone that did receive some endothelial cell damage. However, the central streaks of staining, reported by Ubels et al., 2000, were rarely observed in these preparations. Figure 7 shows a cornea first removed from the holder and then stained. Note the extensive staining at the edge which differs form that seen when the staining occurs in situ. We believe that it is important to evaluate the endothelium under conditions germane to the assay itself. To be absolutely clear, some zones of endothelial cell layer loss (or loss of integrity) are observed in some of the many corneas evaluated histologically. In many cases, this loss comes at the end of the incubation period as indicated by the lack of deep the stromal swelling expected if the loss had occurred soon after mounting. Figure 8 shows the edge of a control cornea incubated for 4 hours. It shows the zone impacted by the O-ring. Note that there is some endothelial cell loss at the edge but no deep stromal swelling.

Figure 5. Bovine cornea mounted in the Spectro Designs cornea holder. The cornea was mounted and incubated for approximately 3 hours. The posterior medium was removed and the corneal endothelium stained with trypan blue. The posterior chamber was then rinsed with medium and the cornea removed from the holder for examination. Note the slight staining at the edge where the cornea was mounted.

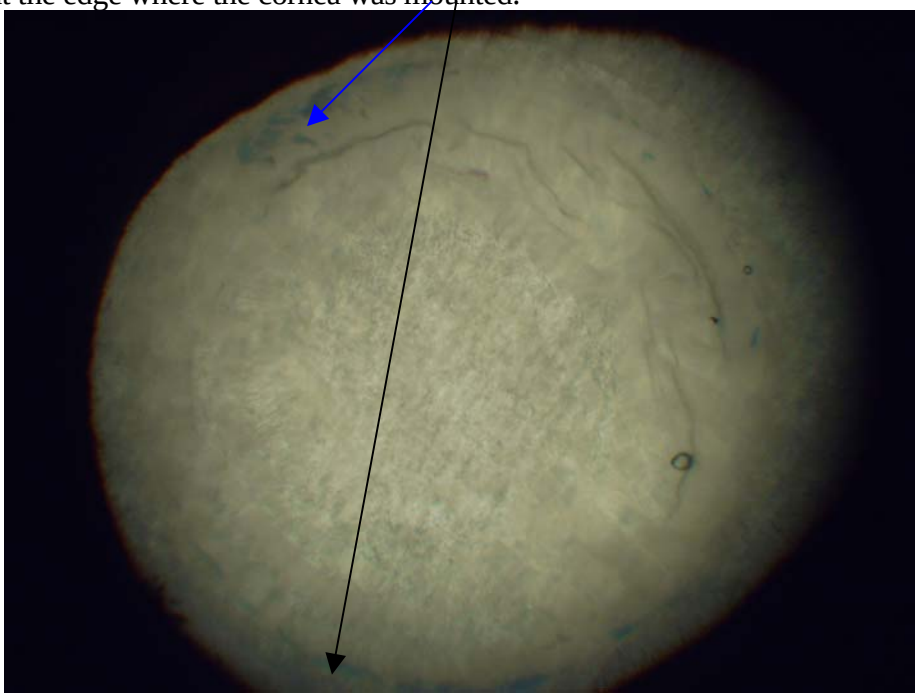


Figure 6. The cornea in Figure 5 showing a patch of damage at one edge.

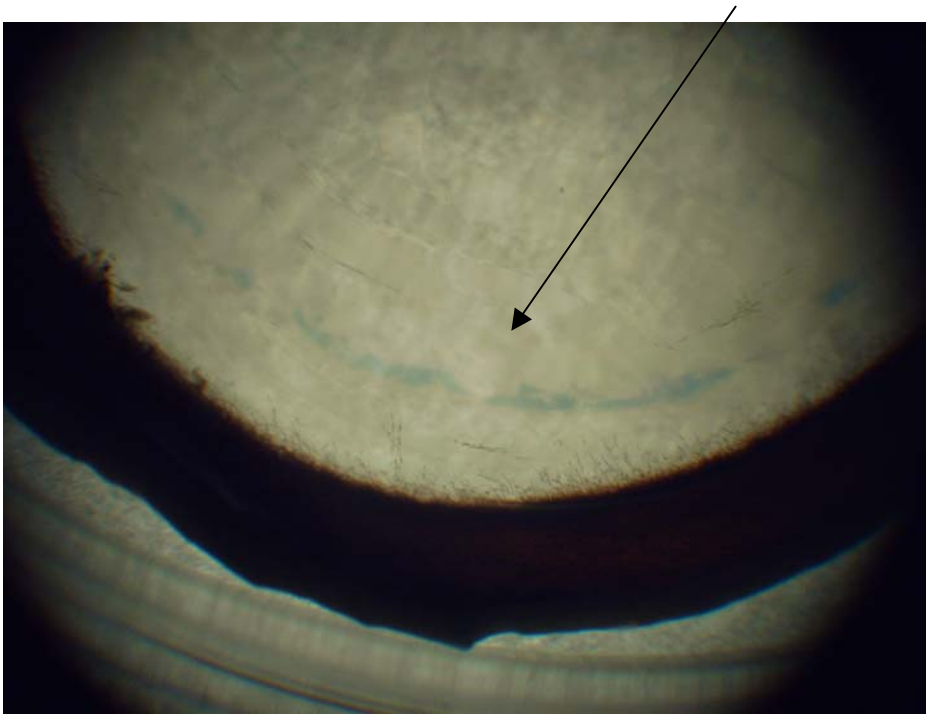


Figure 7. Bovine cornea mounted in the Spectro Designs cornea holder. The cornea was mounted and incubated for approximately 3 hours. The cornea was then removed from the holder and stained with tyrpan blue. Note the extensive damage to the edge of the cornea caused by peeling the cornea off of the holder.

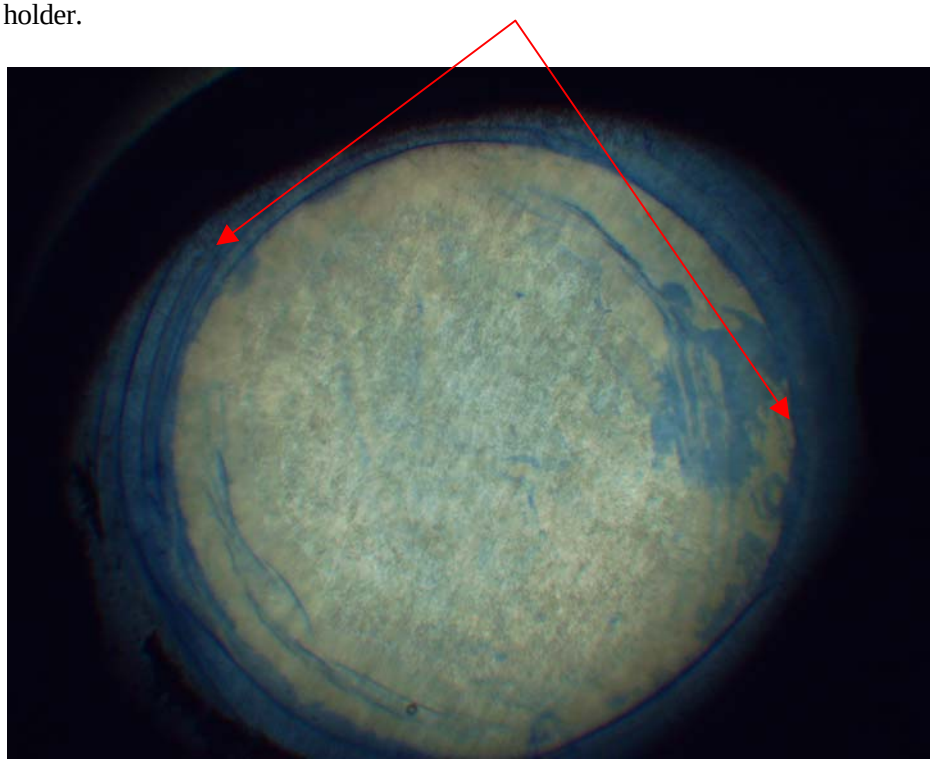
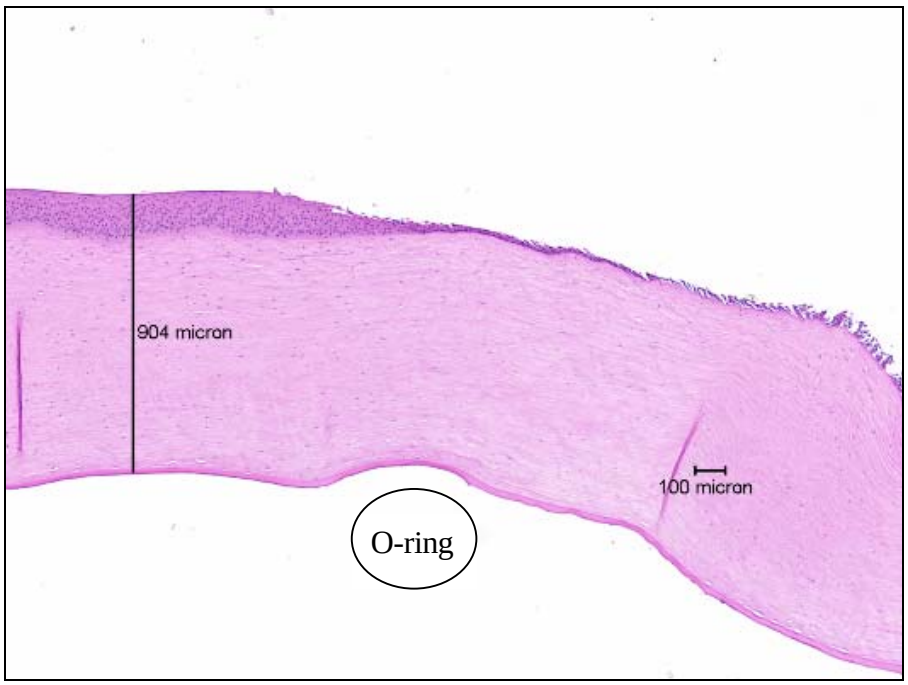


Figure 8. Cross section of edge of a cornea incubated for 4 hours with saline in the anterior chamber (against the epithelium) and medium in the posterior chamber (against the endothelium). The O-ring was located as indicated.



In his written public comments on the BCOP BRD (December 16, 2004), Professor Ubels condemned of the BCOP assay as performed with the Spectro Designs or original Amway corneal holders. He writes in part, "Because the BCOP assay as currently conducted does not meet accepted physiologic standards for studies of isolated corneas I believe that it should not and cannot be validated as alternative toxicological method. Using a flawed alternative method and attempting to validate it with respect to the Draize test, which itself may be criticized on scientific grounds, is not predictive of success in reaching our goal of developing a valid method for testing ocular irritants. The holder currently used must be discarded, and a new data base must be established using methods that do not damage the cornea independently of effects of test materials." We agree with the Expert Panel in their apparent decision not to specifically agree with these comments. In fact, relatively little is known about the proposed holders and how the standard BCOP protocol might need to be modified to account for their structural differences (treatment volume to cover the cornea, treating with solids, volume of the posterior chamber etc). Perhaps when a defined protocol and actual BCOP assay data, from that protocol, become available, all of us will be better able to evaluate the contribution that these holder might make to evaluating eye irritation potential.

7) Testing of Solids (2.1.4) Addressed in section 12 below.

8) Positive control (2.1.7): The positive control is an essential part of every test methods and it should be included in each run of the assay. Ethanol was selected as the positive control because it induced both opacity and permeability in the mid range of responses. However, we concur that a different positive control might be appropriate or at least worthy of review. The positive control substance should be a single chemical (diluted if necessary) rather than a formulation and should induce both opacity and permeability in a range that will allow detection of both hyper-and hypo-responses. To this end, we are evaluating alternative candidates such as dilute benzalkonium chloride.

9) Decision Criteria and the Basis for the Algorithm Used (2.1.13): The "In Vitro Score" was developed to integrate the opacity and permeability scores into a single score for the prediction of irritancy potential (Gautheron et al., 1994 and Sina et al., 1996). However, each measure provides certain information about the mode of action of the test article on the cornea. Therefore, it is useful to examine what each is telling us. For example, mechanical removal of the corneal epithelium leads to a large increase in the passage of fluorescein through the cornea but very little change in opacity (Harbell, unpublished). Coagulation of the epithelium with acid (e.g., trichloroacetic acid) leads to appreciable opacity but no increase in permeability. Surfactants and surfactant-containing formulations that act primarily through membrane lysis (without appreciable protein coagulation) are observed to strip off layers of epithelium and progressively increase the passage of fluorescein without much increase in opacity. Sodium lauryl sulfate (SLS) is an example of such a surfactant (Cater et al., 2001). In contrast, many of the cationic surfactants produce both opacity (coagulation) and increased fluorescein permeability. Casterton et al., 1996, evaluated opacity and permeability separately and used the more aggressive value to predict the

irritation potential. This approach may be particularly appropriate for the range of cleaning products evaluated in these studies. However, our experience with a more diverse range of chemicals and product classes, suggests that the integrated In Vitro Score can provide useful information to complement the examination of each component of the score. No mater which scoring method is selected, the use of concurrent controls (both positive and negative), to establish assay performance, and the concurrent testing of benchmark materials (particularly when dealing with formulations) provides the strongest basis for evaluating irritancy potential. Histology, when performed, should include all of these treatment groups.

Section 3. Substances used for previous validation studies of the BCOP test method

The report raises the question of severe eye damage without corneal opacity. If this note refers to the chronic ulceration of the cornea after recovery from mustard exposure, the point has been addressed earlier in our comments. If there are materials that lead to severe acute eye injury (other than through previous sensitization) without corneal opacity, the panel is requested to provide some examples describing the chemicals and the type of associated eye injury so that these materials might be fully examined in the in vitro assays.

The submission of Swanson et al., 1995, provides examples of heavy-duty cleaning products that were evaluated in vivo as well as in the BCOP assay (please see C-19 to C-22 in the BCOP BRD for formulation details).

Section 4. In Vivo reference data used for an assessment of test method accuracy

We concur wholeheartedly with the panel that the predictive capacity of the reference test must be carefully examined. Clearly, published data are available. It is especially surprising that the seminal publication of the Expert Panel Chair was missing from all of the BRDs. The issue of the predictive capacity of the in vivo test has been at the forefront of the discussion of alternative methods since the early IRAG program.

The ranking of responses by severity is very important to differentiate among inconsistent responses (where only one or two animals show a severe response) and consistent responses (majority or more of the animals show a severe response). This was point raised in the analysis of the CTFA Phase III data (Appendix H5 and specifically H-187), and the public comments of Dr. Curren during the Expert Panel meeting.

It is understood that all test methods show some range of responses across replicate test systems, be they rabbit eyes or isolated organs. Since the in vivo test is scored using a limited series of discreet steps, it is not surprising that, as the damage to the tissue increases, not all animals will manifest a uniform progression to the next grade (e.g., opacity score from 1 to 2). Good laboratories will report the precise responses in each animal independently (see for example Cuellar et al., 2004). We would caution against assuming that apparent uniformity in the reported animal responses from single studies within the same laboratory is indicative that interlaboratory studies (or repeat studies within the same laboratory) will reflect the same consistency. In the majority of Draize tests, animals are not scored blindly; the individual assigned to scoring knows into which chemical test group each animal has been placed. This is in sharp contrast to most in vitro validation studies where the test systems are coded and data are machine read with little or no chance of bias. This concept is particularly important where non-continuous scoring is used.

Section 5. BCOP Test method data and results

The data from Cuellar et al., 2002 (benchmark air care materials and ethanol, in vivo and in vitro) and 2004 (impact of 3-minute exposure and direct comparison of rabbit and BCOP histology) could be useful in the analysis. Both studies were included in the Appendix H but not utilized in the analysis.

Section 6. Test method accuracy

The Expert Panel is to be commended for this discussion (section 6.1) including the minority opinions expressed by Drs. Stephens and Theran. The substitution of "accuracy" for "relevance" implies that the reference method is a nearly infallible standard. Such a change would be a monumental departure for the international consensus for validation developed over the past decade(s)(Balls et al., 1990). The Expert Panel's strong stand and reasoned conclusions – which reversed the conclusions of the original BRD - is very encouraging to the many researchers who view scientific validation as the legitimate path for introducing new approaches for the identification of hazard and the protection of human health.

The question of predicting the irritant potential of alcohols, ketones and solids will be addressed in section 12.

Section 7. BCOP test method reliability

In 7.4, the panel suggests consulting with a range of laboratories to identify constraints that might impact the use of a specific protocol. IIVS certainly concurs with that suggestion and has hosted three workshops on the BCOP assay over the past 7 years to facilitate discussion and exchange.

Section 8. Test method data quality

No comment required

Section 9. Other scientific reports and reviews

Acquiring data from divers sources is key to this program and to future ICCVAM/NICEATM endeavors. The report suggests that the NICEATM made every effort to obtain available data (9.3). We would like to suggest alternative methods of obtaining animal and in vitro data that may be more successful than the current effort. In 1992, the Interagency Regulatory Alternatives Group (IRAG) [predecessor to the ICCVAM] proposed to review the status of alternative tests for the prediction of eye irritation. This group requested data from industry and others without really defining how and by whom the analysis would be conducted. Furthermore, no corporate confidentiality to the submitters was offered. Not surprisingly, little data were proffered. However, in 1993, the program was changed in several important ways. First, data were to be submitted to non-government third

parties (the section chairs). Second, submitters were assured of confidentiality if they so desired. Finally, the specific form of analysis was defined. The data submitted included individual animal tissue scores and the associated in vitro assay data, much as we see today. An impressive body of data was assembled on a wide range of in vitro assays (see Food and Chemical Toxicology, volume 35, 1997).

In contrast, the NICEATM solicited data through Federal Register notices but did not offer third party neutrality, confidentiality or details on the analysis to be performed. Even though considerable data were obtained, we believe that much more additional data could have been received under the right conditions. It is not surprising to us that many organizations are extremely hesitant to submit data from their corporate toxicology programs to a public review that may use a completely different type of analysis, or judgment by different criteria, than the corporations felt was appropriate when developing the program. The Expert Panel might wish to consider how this process could be improved.

Section 10. Animal welfare considerations

No comment required

Section 11. Practical considerations

Training requirements (11.2.2): It is now an established part of any validation study to conduct hands-on training for all participants. It is our experience that there is really no substitute for direct person-to-person training. It is a necessary expense for new laboratories to obtain this training just as new technicians in established laboratories are trained. IIVS has provided this training to third parties in the past and will continue to do so. In point of fact, a BCOP training module will be developed for presentation at the 5th World Congress (post session to be held at ZEBET) in August 2005.

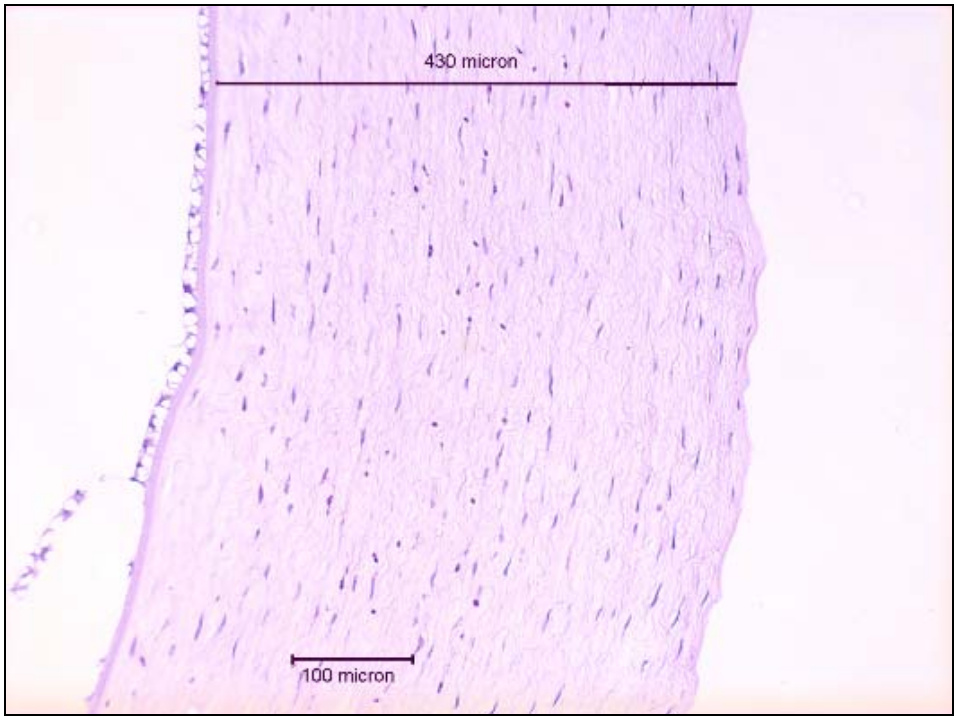
Section 12. Proposed test method recommendations

Testing of alcohols and ketones (organic solvents generally): It is our experience that organic solvents present a number of challenges to a defined exposure system such as the BCOP. In vivo, evaporation, dilution, and flushing of the test article produce an undefined cumulative exposure that may vary from animal to animal and study to study. In the BCOP, the volume, concentration, and time of exposure are fixed. The challenge has been to set the exposure in vitro (specifically, the time variable) to produce similar results to those generally observed in vivo. The results of Balls et al., 1995 suggested that the 10-minute exposure in the BCOP was a greater effective exposure than was generally produced by 100 µL in vivo. Shorter exposures have been evaluated (1, 3, or 5 minutes). Since much of our work has been with alcohols (especially ethanol and ethanol-containing formulations), we have more complete data for this organic solvent.

In the BRD, there are three studies that examine the eye irritation potential of ethanol; the ECETOC 48(2) PAGE 62 (EPA III, GHS 2a (misclassified in the BRD as 2b), Swanson (EPA I and GHS 1 [1/6 did not recover in 21 days]) and Cuellar et al. 2003-2004 (EPA I and GHS 1 [1/1 did not recover in 21 days]). In the studies of Cuellar et al, the impact of solvent on the irritation potential of a defined fragrance oil formulation was

evaluated. The solvents were also tested alone. Ethanol was one of the solvents tested (for compete details please see Appendix H-1). Generally, four rabbits were treated and evaluated using the normal Draize protocol. At the end of the 24-hour evaluation period, the rabbit eyes were taken for histology from three of the four animals treated. The fourth animal was followed to recovery or to day 21. Figure 9 shows the cross section of one of the ethanol treated corneas. Note the complete loss of epithelium, decrease in the density of viable keratocytes in the upper stroma, and keratocytes with enlarged nuclei and more eosinophilic cytoplasmic staining. Inflammatory cells are also present. The loss of the epithelium, itself, may not be sufficient to cause the severe responses observed in the minority of rabbits treated with ethanol (taking all studies in total), but possible variations in stromal injury may contribute to the differences. The loss of epithelium may open individual animals to secondary infections (M. Prinsen, personal communication).

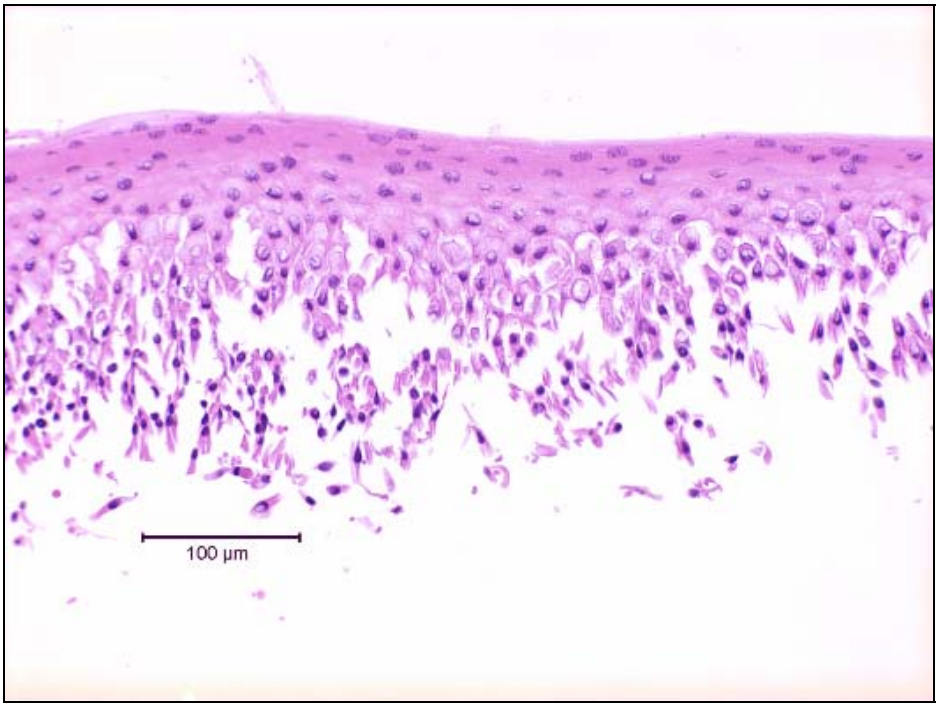
Figure 9. Ethanol-treated in vivo 24 hours before harvest: Central cornea showing loss of epithelium, inflammation, and marked increase in larger dark staining keratocyte nuclei in area of inflammatory infiltrate.



In the ethanol-treated eyes, appreciable coagulation in the stroma was not evident. The endothelium was not well preserved in these sections but the changes were also observed in the contra lateral, untreated eyes (suggesting an artifact of fixation/processing). At 24 hours after treatment, four of the five animals treated showed 2x4 opacity scores. One animal showed only a slight conjunctival response at 1-hour post instillation. The weakly responding animal was harvested at 24 hours along with three of the four responding animals. No corneal lesions were observed in the weakly responding animal.

The same treatment groups were evaluated in the BCOP assay using exposure times of 1 minute with a 20-hour post-exposure incubation and 3 minutes with post-exposure periods of 2, 4, and 20 hours. These post-exposure times refer to the time, after removal of the test or control articles, that the final opacity value is taken and the fluorescein added. The actual fixation of the corneas comes after the 90-minute incubation with fluorescein. The 10-minute exposure to ethanol was the positive control for this study (2-hour post exposure). The 1-minute exposure lifted the epithelium from the cornea. This allowed the stroma to swell as expected. Both changes were consistent with the action in vivo (Figure 10). However, several of the other treatment groups were under-predicted by this short exposure. Therefore, focus was turned to the 3-minute exposure.

Figure 10. Epithelium after ethanol treatment (1-minute exposure and 20-hour post-exposure)



Figures 11 to 13 show the bovine corneal epithelium 2, 4 and 20 hours after a 3-minute exposure. Compared to the 1-minute exposure, the squamous epithelium in more coagulated and the epithelium tends to separate from the Bowman's Layer as a complete sheet. The stromal changes are more limited at 2 hours post-exposure compared to the rabbit eye after 24 hours. However, by 4 hours post-exposure, the upper stromal swelling, decrease in keratocyte density and appearance of cells with eosinophilic staining cytoplasm (in the deeper stroma) is complete. Figure 14 shows an example of a bovine cornea treated for 10 minutes with ethanol followed by a 2-hour post-exposure (standard positive control conditions). Under these conditions, the epithelium is not appreciably different from that of the 3-minute exposure but the upper stroma shows loss of viable keratocytes (and some

possible collagen coagulation) as well as the enlarged keratocytes with eosinophilic cytoplasmic staining (Figure 15).

Figure 11. Epithelium and upper stroma of a bovine cornea treated with ethanol for 3 minutes and incubated for 2 hours post-exposure (before fluorescein addition)

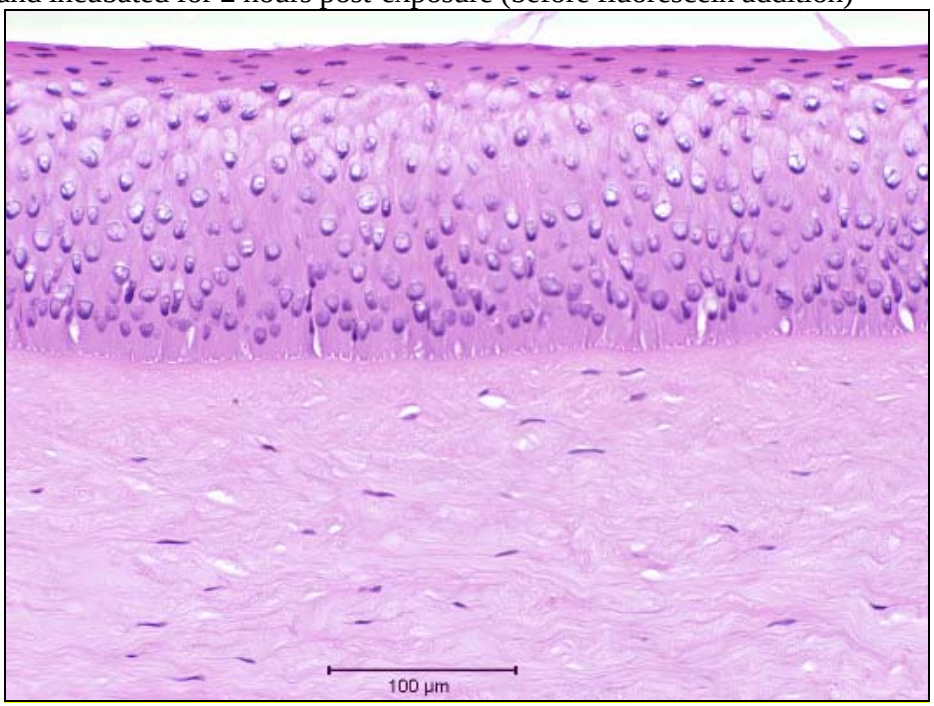


Figure 12. Epithelium and upper stroma of a bovine cornea treated with ethanol for 3 minutes and incubated for 4 hours post-exposure (before fluorescein addition)

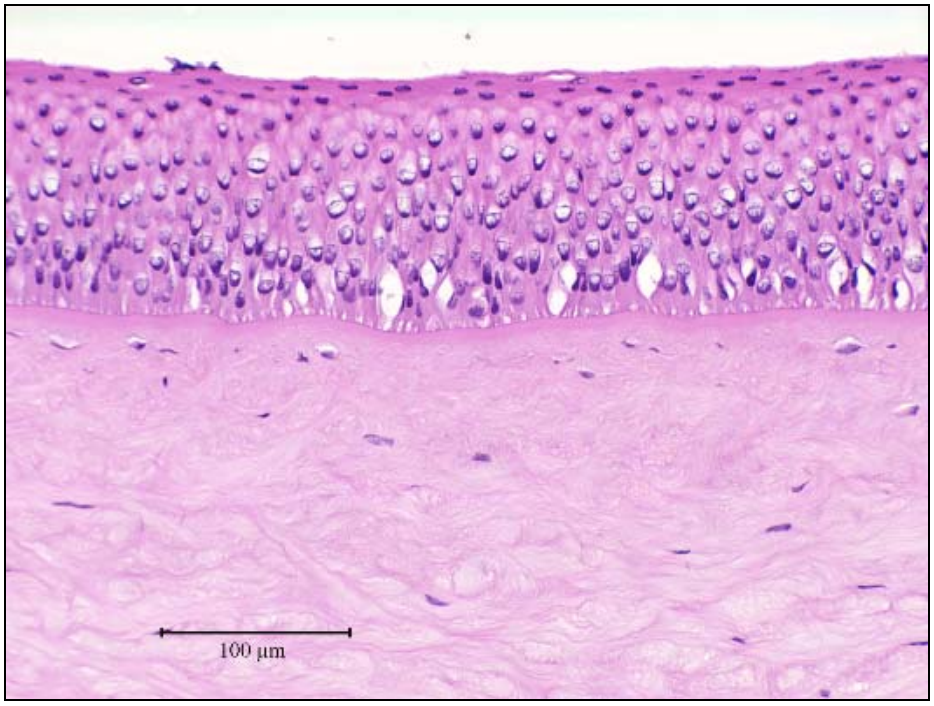


Figure 13. Epithelium and upper stroma of a bovine cornea treated with ethanol for 3 minutes and incubated for 20 hours post-exposure (before fluorescein addition)

Figure 14. Epithelium and upper stroma of a bovine cornea treated with ethanol for 10 minutes and incubated for 2 hours post-exposure (before fluorescein addition)

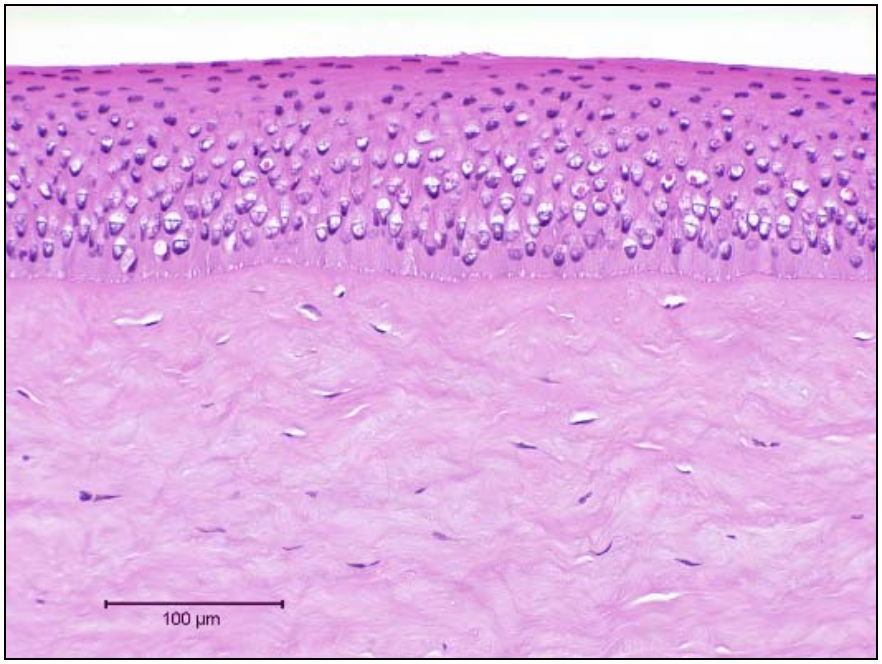
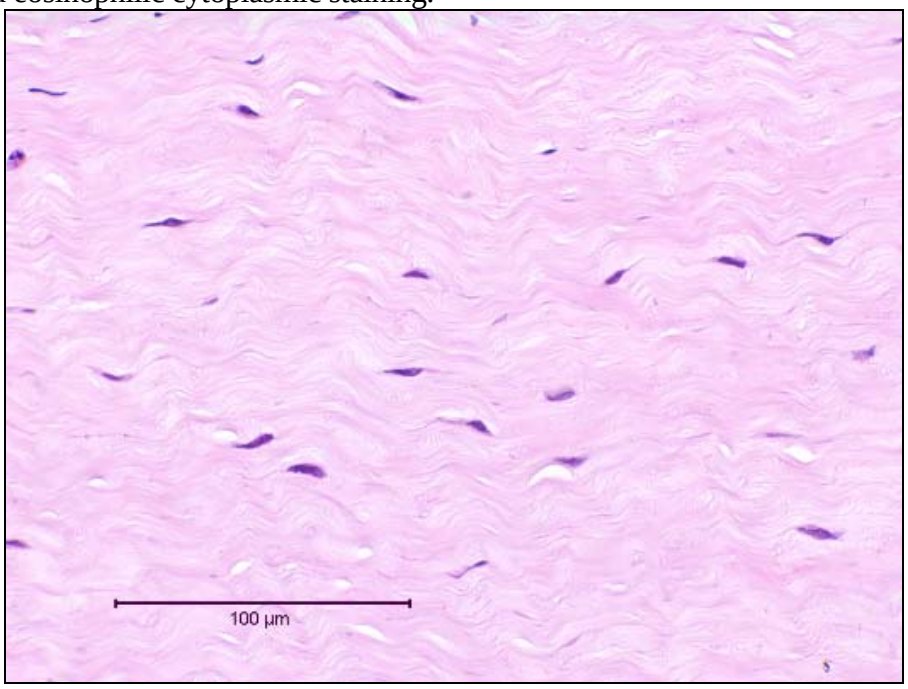


Figure 15. Stroma below mid depth of a bovine cornea treated with ethanol for 10 minutes and incubated for 2 hours post-exposure (before fluorescein addition). Note the enlarged nuclei and eosinophilic cytoplasmic staining.



At IIVS, the standard approach to testing organic solvent-containing formulations (such as the air care products discussed in Cuellar et al., 2002 [Appendix H2]) has been to exposure the test article and benchmark formulations to the corneas for both 3 and 10 minutes (three corneas each). The goal in this approach is to identify those formulations that induce an increased opacity/permeability after only a short exposure compared to those that require a longer exposure. The benchmark formulations facilitate interpretation. We believe that this approach provides sound data for predicting the eye irritation potential of alcohol/organic solvent-containing formulations as well as for the as the solvents themselves.

The panel raised concerns about the inter-laboratory consistency of the response to ethanol in the prevalidation study of Southee et al (1998). While we cannot speak to specific factors in that study, we are aware of several factors that can impact the response to ethanol (and probably many of the organic solvents). The first is the temperature of the ethanol when it is added to the chambers. While the chambers themselves are incubated at 32ºC, the test and control articles are held at “room temperature” before addition (as they are when tested in vivo). It is our experience that warm ethanol will produce a greater increase in opacity/permeability than will cool ethanol even through the holders are at 32ºC. The second factor is the potential sloughing of portions of the epithelium. It is the coagulated epithelium that contributes the bulk of the opacity value. Since the opacitometer is “center-weighted” in its reading, loss of portions of the epithelium from the central cornea will decrease the opacity value. However, they seem to increase the passage of fluorescein so that there can be some balancing between the two factors. We believe this is why the CVs of the opacity and permeability scores exceed the CV of the In Vitro Score (please see page H-58, BRD).

Testing of solids: The BCOP assay was developed by Drs. Gautheron and Sina specifically to address the testing requirements of pharmaceutical intermediate compounds that are often, if not generally, poorly water soluble or non-water soluble solids. This type of test material was evaluated in Gautheron et al. 1992, VanParys et al., 1993, and Sina et al., 1995. Therefore, it comes as some surprise to us that the panel report would be forced to question the use of the assay for solids. This concern probably reflects the limited data available to the panel. Much of the pharmaceutical intermediate animal data could not be retrieved, in the required form, in time for the BRD preparation and so data on solids was limited to those supplied through Balls et al., 1995 and Gautheron et al., 1994. These totaled approximately 33 chemicals (with some overlap between studies). Of these, seven materials showed under-prediction or markedly inconsistent prediction in the BCOP compared to the animal data provided. This was a concern at the time to many users of the BCOP assay. Therefore a study was undertaken at IIVS to re-evaluate many of the least well-predicted chemicals. Not all of the under-predicted chemicals were available and some materials with good in vitro predictions were also included. Two additions to the protocol were included. First, a 16-hour post-exposure was included in addition to the standard 2-hour post-exposure (for liquids) and 4-hour exposure for solids. Second, corneas were fixed and processed for histological evaluation.

Several conclusions were drawn from this study. First, the in vitro data generated in our laboratory were almost identical to that generated by the participants in the EU/Home Office study several years before. Second, the 10-minute exposure period for organic solvents produced more damage than was expected from the in vivo scores and days to clear (e.g., toluene and n-butyl acetate)(as discussed above). Third, certain of the markedly under-predicted solid test articles manifested clear lesions (depth of injury) when evaluated histologically (sodium oxalate, aspartic acid, and quinacrine). Some of these lesions required the longer post-exposure incubation to be fully manifested. This observation is consistent with the delayed onset of maximal response in vivo. Because of this study, we recommended that new/unknown chemicals be evaluated with a revised protocol to account for delayed onset of toxicity and development of lesions that do not result in appreciable increases in opacity or permeability scores (e.g., quinacrine which gave an in vitro score of 4.4 in our hands). The same approach is taken with peroxide-containing formulations. For such test materials, we recommend the normal exposure specified by the protocol (depending on the physical state of the material), but a 4-and 20-hour post exposure with corneas collected from both time points for histology. We believe that the addition of histology, as recommended by the Expert Panel, will significantly reduce the chances of false negative results with test materials in general and solids in particular. The addition of additional existing data sets will also broaden the evaluation.

Section 12.2 Minority Opinions: In our preceding comments, we have tried to address certain technical points contained in the BCOP chapter. We would like to strongly emphasize our appreciation to the members of the Expert Panel subgroup who spent considerable energy in preparing the review of the BCOP assay. However, we also wish to address the process of the Expert Panel review. We agree with the minority opinions of Drs. Freeman, Stephens, and Theran and believe these opinions are a tremendously important

contribution to this Expert Panel Report and to the success of new toxicological test method evaluations by future Expert Panels.

It was not clear to many of us observing the recent process how the final validation decision was going to be made. The role of the Expert Panel relative to the role of the Ocular Toxicology Working Group and the entire ICCVAM committee was confusing. We believe that there needs to be a much clearer definition of how validation decisions will be made, and what the relative input of various groups will be. This is of fundamental importance to a process that is extremely important to many stakeholders. As befits such an important process, we believe that the Scientific Advisory Committee for Alternative Toxicological Methods should have a significant involvement in decisions on how the process of validation assessments occurs, and what the roles of various review groups, NICEATM, and ICCVAM are. Once again, we feel that the minority opinions of the three Panel members document the need to provide clear, complete and neutral guidance to an Expert Panel from the onset of the review process, and to allow the Panel to state and maintain its conclusion in a free and open professional atmosphere.

In section 12.3.2, the Expert Panel has suggested additional data be made available to support the 3-minute exposure time for volatile solvents. We have tried to present an overview here and agree that it is important to make such data available for review by the scientific community. Much of this information has already been presented in other forms. The testing of solids is a slightly different issue. Given the large body of data available from BCOP assays on the pharmaceutical intermediate chemicals, the issue seems to one of identifying those materials that might not produce opacity or permeability changes that reflect the degree of damage induced. We believe that the addition of histology to the evaluation would address that point.

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