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HET-CAM for the identification of ocular irritancy in seawater/salt water sources

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HET-CAM FOR THE IDENTIFICATION OF OCULAR IRRITANCY IN SEAWATER/SALT WATER SOURCES

by

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ABSTRACT

The Hens Egg Test on the Chorionallantoic Membrane (HET-CAM) is an ocular irritation test proposed to replace tradition ocular investigations such as the Draize eye test that has been criticized because of its use of animals. In this study, the ocular irritation potential of seawater and saltwater sources dosed with different disinfectants has been evaluated using the HET-CAM. The seawater and salt water tested was taken from the main show pools from three aquatic theme parks (San Diego, San Antonio, and Orlando). The three disinfectants considered were sodium hypochlorite (NaOCl), calcium hypochlorite ($Ca(OCl)_2$), and ozone (O_3).

Three different HET-CAM methods were performed. Egg preparation using *Method One* proved to be very difficult, and very few signs of irritation were observed. Egg preparation using *Method Two* proved much easier, but the new setup helped induce the irritation observed. Finally, egg preparation using *Method Three* was similar to *Method Two*, but alleviated the affect of irritation induced by outside factors.

The development of a working method left little time to obtain pertinent results. Nevertheless, as suggested by Erdinger et al., a synergistic effect was observed and may contribute to the ocular irritation induced. Also, the ocular irritation potential of $Ca(OCl)_2$ appeared to be mildly less offensive than CaOCl.

Ultimately, the results at hand do help to serve as a guideline for anyone who may wish to pursue this project/method further.

ACKNOWLEDGEMENTS

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1. INTRODUCTION

Traditionally, U.S. Federal and international regulatory agencies have performed investigations of chemical ocular irritancy using the Draize eye test, which applies test chemicals directly on the eyes of rabbits. The test solution is applied to one eye of a rabbit, leaving the other to serve as the negative control. The rabbit's eyes are then observed over 21 days to identify reversible or irreversible adverse effects to the conjunctiva, iris, and cornea [1].

There has been strong support for the development of new, more ethical, tests [2]. The EPA requested evaluations of four different in-vitro ocular irritancy tests: the Isolated Chicken Eye (ICE), the Isolated Rabbit Eye (IRE), the Hen's Egg Test on the Chorionallantoic Membrane (HET-CAM), and the Bovine Corneal Opacity and Permeability (BCOP) [1].

One such in-vitro replacement to the Draize eye test is the HET-CAM, developed by Luepke, in which the chorionallantoic membrane (CAM) of embryonated hens' eggs is used to test possible ocular irritants. The CAM is a vascular fetal membrane, composed of the fused chorion and allantois. The CAM is made up of three layers: an ectodermal layer (the white layer seen after removing the shell) consisting of a two- to three-cell thick epithelium; a mesodermal layer consisting of connective tissue, ground substance, and blood vessels; and an endodermal layer. The small blood vessels and proteins of the soft tissue membrane are thought to respond to acute effects induced by test substances. The test is based on the idea that the CAM's response and makeup correlate with those of the vascularized mucosal tissues of the rabbit eye [1].

In comparison with structures of the human eye, the CAM, though thinner and in combination with a less advanced ectodermal layer, is most similar to the conjunctiva; as both are mucous membranes with a functioning vascular system. The reaction to irritants by the CAM and the conjunctiva are quite different though. The conjunctiva accumulates macrophages (immune cells that devour invading pathogens) and experiences neutrophil (a type of white blood cell) infiltration, both imperatives in inflammation response, whereas the CAM experiences cell death in the area of application [1].

Currently, the HET-CAM method is being used in U.S. and E.U. companies that fabricate pharmaceuticals and cosmetics. The in-vitro test has yet to be validated for distinguishing between eye irritants and non-irritants, although in the E.U., positive tests for severe irritation are accepted. Following a negative result using the HET-CAM test for eye irritancy, subsequent tests, often in-vivo, are pursued [1].

In a previous proposal for Busch Entertainment, it was reported that trainers and animals (e.g., whales and dolphins) experienced acute and chronic ocular irritation from show and back basins in all three aquatic theme parks: California, Florida, and Texas. Irritation reports were highly variable between trainers.

It was hypothesized that the ocular irritation may be induced by haloamines (e.g., chloramines and/or bromamines), organic disinfection byproducts (e.g., haloacetic acids, organic amines, halomethanes), other inorganic disinfection byproducts (e.g., bromate), or possibly algal toxins released from algal or cyanobacteria. The chlorination and ozonation chemistries of seawater (natural) and salt water (artificial) are complex due to the concentrations of inorganics (e.g., chloride and bromide ions) and organics (e.g., natural organics, animal fecal matter, and urea).

The aquatic theme parks commonly use free chlorine and ozone disinfection (i.e., control of bacteria and algae). Both oxidants inactivate pathogenic organisms differently. Ozone is thought to directly oxidate/destruct the cell wall, leaking cellular constituents outside of the cell, as well as damage the constituents of the nucleic acids (purines and pyrimidines) [3]. The disinfection/inactivation mechanism of chlorine is not well understood [4]. A few proposed mechanisms include DNA repair enzyme loss, base-pair mutation, single and double strand breakage, as well as targeting of the cell envelope [5,6].

Ozone is beneficial for the parks because it also serves as a microflocculant and reduces the appearance of color through oxidation. However, both free chlorine and ozone create secondary oxidants and other disinfection byproducts through their reactions with organic and inorganic water constituents.

Furthermore, ocular irritation information is limited in seawater/salt water systems. Chloramines are suggested to be primary ocular irritants (e.g., trichloramine (NCl_3), dichloramine (NCl_2H), and monochloramine ($NClH_2$)). Ocular irritation effects of monochloramine and free chlorine (hypochlorous acid (HOCl) and hypochlorite (OCl^-)) may be very minor. The formation of chloramines is achieved by the reaction of free chlorine and ammonia/ammonium (NH_3/NH_4^+):

$$HOCl/OCl^- + NH_3/NH_4^+ \rightarrow NClH_2 \quad (1)$$

$$HOCl/OCl^- + NClH_2 \rightarrow NCl_2H \quad (2)$$

$$HOCl/OCl^- + NCl_2H \rightarrow NCl_3 \quad (3)$$

Animal excretions provide the ammonia in the basins. Free chlorine is added for water disinfection, but is also formed by the reaction of ozone (O_3) with the high concentrations of chloride ion (Cl^-) in salt water or seawater.

Bromamines are formed by the reaction of ammonia with free bromine (hypobromous acid (HOBr), and hypobromite (OBr^-)). The reaction of free chlorine with the bromide ion, a constituent of salt water or seawater, readily form free bromine. Thus, during both chlorination and ozonation of seawater/salt water, free bromine and bromamines can be formed.

Erdinger et al. performed a study of the effects of different halogenated carbonyl compounds using the HET-CAM. They found that the compounds under investigation were only irritants at concentrations much higher than what is typically found in swimming pools. However, when the compound under investigation was accompanied by an oxidant, the irritation concentration of the compound was decreased significantly (factor of ten). They determined that the irritating effect experienced in swimming pools was not based on a single compound, but rather, synergistically. The oxidants used to treat swimming pools are very reactive, and the byproducts formed through oxidative pathways contribute to the effect.

Similar tests were performed here. Doses of single compounds as well as multiple compounds (synergistic effect) were applied to chorionallantoic membranes.

Four different HET-CAM procedures were reviewed from the literature.

1.1 HET-CAM METHOD BY LUEPKE [7]

Fertile white Leghorn eggs are incubated at 37.5°C ± 0.5 with relative humidity at 62.5% ± 7.5. Eggs are candled on the fifth day of incubation and every day thereafter, and defective eggs are discarded. On the tenth day of incubation, the shells are removed above the air pockets and the ectodermal membranes are removed. A volume of 0.2ml of testing material is applied directly onto each CAM. The eggs are again placed into the incubator, and the CAMs are observed over 5 minutes for vascular lysis (blood vessel disintegration), hemorrhage (bleeding from the vessels), and coagulation (intra- and extra-vascular protein denaturation).

The numerical time-dependent scores for vascular injection, hemorrhage, and coagulation are totaled to give a single numerical value (Table 1.1.). This value indicates the irritation score (IS) of the test substance with a maximum value of 21. The mean value of, in minimum, 4 tests is taken, and the test substance is classified according to Table 1.2.

Table 1.1. HET-CAM Scoring System

	0.5min	**2min**	**5min**
Effect		**Score**	
Vascular injection	5	3	1
Hemorrhage	7	5	3
Coagulation	9	7	5

Table 1.2. HET-CAM Scoring Assessment

Cumulative Score (mean)	**Assessment**
0 to 0.9	Practically no irritant
1 to 4.9	Slight irritant
5 to 8.9	Moderate irritant
9 to 21	Strong irritant

1.2 HET-CAM METHOD BY SPIELMANN [8]

Fertile white Leghorn eggs (50-60g) are incubated at 37.5°C ± 0.5 with relative humidity at 62.5% ± 7.5. Eggs are incubated on their sides and rotated for eight days, when rotation ceases. Eggs are candled on day nine, defective eggs are discarded, and workable eggs are again placed into the incubator, with the large ends up.
On day ten, eggs are candled and prepared. The shells around the air pockets are removed, the ectodermal layers are moistened with 0.9% NaCl, and the eggs are placed in the incubator, large ends, up for no longer than 30 minutes. Afterwards, the eggs are removed from the incubator, the 0.9%NaCl is decanted, and the ectodermal layers are removed with forceps.

A volume of 0.3ml of the test solution is applied directly onto each CAM. The CAMs are then observed over 300 seconds for hemorrhage, vascular lysis, and coagulation. The time for each is recorded, and the IS is determined according to Eq.1. The test substance is classified according to Table 1.3.

$$[(301-H)/300]x5 + [(301-L)/300]x7 + [(301-C)/300]x9 \quad (4)$$

Table 1.3. IS Method Analysis Classification Schematic

HET-CAM Score Range	Irritation Category
0 to 0.9	Nonirritant
1 to 4.9	Slight Irritation
5 to 8.9	Moderate Irritation
9 to 21	Severe Irritation

1.3 HET-CAM METHOD BY NICEATM-ICCVAM REPORT: APPENDIX G (2006) LUEPKE [2]

Fertile white Leghorn chicken eggs (not older than 7 days, and between 50-80 grams) are incubated at 38.3°C ± 0.2 and a relative humidity of 58% ± 2. Defective eggs, excessively misshapen, cracked, etc., are discarded. Workable eggs are hand rotated five times per day until day eight when they are candled and checked for viability. Once again, defective eggs are discarded. Workable eggs are then returned to the incubator, large ends up, without further rotation until day nine.

On day nine, the air cells, observed by candling, are marked and cut away. The ectodermal layers are moistened with 0.9% NaCl, and the eggs are replaced into the incubator large ends up for no longer than 30 minutes. Afterwards, the eggs are removed from the incubator, the 0.9% NaCl is decanted, and the ectodermal layers are removed with forceps. A volume of 0.3ml of liquid or diluted substances is applied directly on the CAMs. The CAMs are then observed over 300 seconds for hemorrhage, vascular lysis, and coagulation. The time for each is recorded, and an IS is determined (Eq. 4). Where H = start time of hemorrhage; L = start time of vascular lysis; C = start time of coagulation. The IS is determined by Eq. 4, and the test solution's classification is determined by Table 1.2.

The test is considered acceptable if the negative and positive controls each induce a response that falls within the classification of nonirritating and severely irritating. Positive controls, 1% SDS or 0.1N NaOH, typically produce an IS value of 10 and 19, respectively. Negative controls, 0.9% NaCl, typically produce an IS value of 0.0.

1.4 HET-CAM METHOD BY ERDINGER, KIRSCH, AND SONNTAG [9] *(POSSIBLE GERMAN TO ENGLISH MISTRANSLATIONS)*

Fertile white Leghorn chicken eggs are incubated at 37°C, with relative humidity at 63% and incubated for nine days. At nine days, the air cells are cut away 2mm above the membranes. Closely above the membranes, a small hole is drilled into the eggshell. Next, the ectodermal membranes are removed with tweezers, and the testing material, initially dissolved in DMSO, is put into an infusion bottle that carefully feeds it onto each CAM at 1.25mL/min. Every test is done with three eggs in parallel, and the entire procedure is performed in the incubator at 37°C. (The lowest concentration applied in a testing series is measured by gas chromatography).

The CAMs are observed over one hour for hyperemia (expansion of vessels), vascular lysis (vessel disintegration), hemorrhage (bleeding from the vessels), coagulation of protein, and coagulation of blood. Testing material is classified as *Weakly Irritating* if only weak symptoms such as hyperemia are observed. The classification is *Moderately Irritating* if, in addition, single appearances of more severe irritations such as hemorrhage, vascular lysis or coagulation are observed. Lastly, the classification is *Severely Irritating* if more than one moderately irritating effect is observed.

2. OBJECTIVES

The HET-CAM method is being performed in cooperation with an aquatic theme park and the Environmental Research Center of Missouri University of Science & Technology (MS&T) in order to identify compounds that induce eye irritation in both animals and trainers.

The HET-CAM is proposed to be the screening method to be used to compare the relative ocular irritation caused by chlorination and ozonation of saltwater versus seawater over a range of doses, chloramines and bromamines directly, algal toxins (if any) are found, and additional halogenated disinfection byproducts including haloacetic acids (HAAs).

This report focuses on the saltwater samples from the parks experiencing ocular irritation, and their chlorination and ozonation. Chloroform and bromoform were also considered.

3. METHODS AND MATERIALS

The HET-CAM was being used to investigate possible ocular irritants in saltwater sources from aquatic theme parks. Recently, both animals and trainers have experienced ocular irritation from the saltwater or seawater used in these parks. They asked the Environmental Research Center of the University of Missouri Science & Technology (MS&T) to investigate the sources and culprits causing the irritancy, as well as possible monitoring and corrective options. The irritation was suspected to be a result of the reaction of the saltwater, either artificial or taken from nearby saltwater bodies (seawater), the oxidants, and the animal waste to produce such compound groups as, but not limited to, haloamines (HAs), haloacetic acids (HAAs), and trihalomethanes (THMs).

The HET-CAM was proposed to be the screening method to compare the relative irritation caused by chlorination and ozonation of saltwater versus seawater over a range of doses, chloramines and bromamines directly, algal toxins (if any) are found, and additional halogenated disinfection byproducts, including HAAs.

This report focused on the saltwater and seawater samples from the parks experiencing ocular irritation, and their chlorination and ozonation. Chloroform and bromoform were alsoconsidered.

The oxidants considered were free chlorine (sodium hypochlorite and calcium hypochlorite) and ozone. Concentrations of disinfection solutions were determined by HACH Method 80 for Free Chlorine. Ozone was created with a PCI-WEDECO (Environmental Technologies) Ozone Generator. The ozone concentration was found spectrophotometrically (λ=260).

3.1 METHOD ONE

Fertilized white Leghorn chicken eggs from Moyer's Chicks, Quakertown, PA, were placed in an Octagon 40 Digital incubator from Brinsea Products Inc. with full automatic egg turning and a side humidity module (also from Brinsea Products, Inc.) at 37.4°C ± 0.2 and relative humidity at 61% ± 4% [3]. Eggs were incubated for nine to ten days [2,7,8]. Further, they were left undisturbed for 48 hours after placement in the incubator and were subject to candling for viability at any point afterwards. (Candling is the process of shining light through an egg in order to check for proper development of the embryo.)

Candling was performed by placing a floodlight under an overturned, clay flowerpot, which allowed light to escape through the small hole on its bottom. This contraption was then placed under a cardboard box with three holes cut out: one in front for the eyes, and one on either side for arms to enter. The box helped create a darker environment than what the lab would normally allow.

On the ninth or tenth day, eggs were removed for the HET-CAM [2,7,8,9]. Next, each egg was candled to identify the air pocket near the top. Using a Dremel Cordless Rotary Tool with a cut-off wheel, the shells were cut around the air pockets identified by a pencil tracing. Once the shells were removed, the white ectodermal membranes were painstakingly removed with tweezers and a dental explorer. The removal of these membranes exposed the chorionallantoic membranes.

Test substances were applied to each chorionallantoic membrane in a volume of 0.3ml [2,8]. Pictures were taken of each membrane before application, just after the

application, and at 30 sec, 1 min, 2 min, 3 min, 4 min, and 5 min intervals with a D50 Nikon Digital Camera with DX Nikon zoom lens sitting atop a camera stand [2].

Oxidation runs, using free chlorine (NaOCl) or ozone, were performed immediately after the oxidant spike was introduced to each sample, and also after the saltwater, or seawater, and oxidant had been in contact for 30 minutes.

Test substances from "Method One HET-CAM Experimental Outline" (Table 3.1.) were scored according to Luepke, which assesses the speed and severity of the damage observed to the small blood vessels and proteins of the soft tissue membranes (Table 1.1. and Table 1.2.).

3.2 METHOD TWO

Egg development was done exactly as stated in *Method One*. The procedure was performed inside the incubator at developmental specifications (37.4°C ± 0.2 and relative humidity at 61% ± 4%) to ensure that the eggs did not cool and that there was no reduction in blood flow [9]. The inner membranes were saturated with 0.9%NaCl and placed back into the incubator for no longer than 30 minutes. Next, the NaCl solutions were decanted and the inner membranes were removed to expose the CAMs. Performed in triplicate, test substances were applied to the chorionallantoic membrane of each egg in a volume of 0.3ml and observed for 1 hour. Pictures were taken of the membrane before application, at 5 min, 10 min, 20 min, 30 min, 40 min, 50 min, and 60 min intervals with a D50 Nikon Digital Camera with DX Nikon zoom lens sitting atop a camera stand.

Oxidation runs, using free chlorine (NaOCl or $Ca(OCl)_2$) or ozone, were performed immediately after the oxidant spike was introduced to each sample.

Table 3.1. Method One HET-CAM Experimental Outline

Run	Sample[a]	Oxidant[b]	Oxidant Dose (mg/L)	Time after Oxidant Spike (min)	Note
1	+ Control	NA	NA	NA	1%NaOH
2	- Control	NA	NA	NA	0.9%NaCl
3	OLMB	NA	NA	NA	CONTROL
4	OLMB	Cl_2	3	1	
5	OLMB	Cl_2	3	30	
6	OLMB	O_3	3	1	
7	OLMB	O_3	3	30	
8	SDMB	NA	NA	NA	CONTROL
9	SDMB	Cl_2	3	1	
10	SDMB	Cl_2	3	30	
11	SDMB	O_3	3	1	
12	SDMB	O_3	3	30	
13	TXMB	NA	NA	NA	CONTROL
14	TXMB	Cl_2	3	1	
15	TXMB	Cl_2	3	30	
16	TXMB	O_3	3	1	
17	TXMB	O_3	3	30	
18	chloroform	NA	NA	NA	100ppm
19	bromoform	NA	NA	NA	200ppm

[a] The first two letters of the abbreviations under the sample heading refer to the aquatic theme park, while the last two refer to the pool within the site being tested (OL-Orlando, SD-San Diego, TX-Texas; MB-Main Basin). [b] NA - not available, Cl_2 – NaOCl as Cl_2.

Concentrations of free chlorine and ozone were determined with HACH Method 80 for Free Chlorine and spectrophotometrically, respectively.

The CAMs were observed for hyperemia (expansion of vessels), vascular lysis (vessel disintegration), hemorrhage (bleeding from the vessels), protein coagulation, and/or blood coagulation [9].

Test material was classified as *Weakly Irritating* if only weak symptoms (e.g. hyperemia) were observed. Its classification was *Moderately Irritating* if, in addition, single appearances of more severe irritations (e.g. hemorrhage, vascular lysis or

coagulation) were observed. Lastly, it was classified as *Severely Irritating* if more than one moderately irritating effect was observed [9].

Test substances from "Method Two HET-CAM Experimental Outline" (Table 3.2.) were scored according to Erdinger et al.

Table 3.2. Method Two HET-CAM Experimental Outline

	Sample[a]	Oxidant	Dose (mg/L as Cl_2)		Sample[a]	Oxidant	Dose (mg/L as Cl_2)		Sample[a]	Oxidant	Dose (mg/L as Cl_2)
1	SDMB	NaOCl	10	10	OLMB	NaOCl	10	19	TXMB	NaOCl	10
2		NaOCl	100	11		NaOCl	100	20		NaOCl	100
3		NaOCl	1000	12		NaOCl	1000	21		NaOCl	1000
4	SDMB	$Ca(OCl)_2$	10	13	OLMB	$Ca(OCl)_2$	10	22	TXMB	$Ca(OCl)_2$	10
5		$Ca(OCl)_2$	100	14		$Ca(OCl)_2$	100	23		$Ca(OCl)_2$	100
6		$Ca(OCl)_2$	1000	15		$Ca(OCl)_2$	1000	24		$Ca(OCl)_2$	1000
	Sample	Oxidant	Dose (mg/L)		Sample	Oxidant	Dose (mg/L)		Sample	Oxidant	Dose (mg/L)
7	SDMB	O_3	10	16	OLMB	O_3	10	25	TXMB	O_3	10
8		O_3	100	17		O_3	100	26		O_3	100
9		O_3	1000	18		O_3	1000	27		O_3	1000

[a] The first two letters of the abbreviations under the sample heading refer to the aquatic theme park, while the last two refer to the pool within the site being tested. (OL-Orlando, SD-San Diego, TX-Texas; MB-Main Basin)

The concept behind this experimental outline was to identify each oxidant's concentration range that produced ocular irritation and, if time permitted, to refine the experiment to narrow down that concentration.

3.3 METHOD THREE

Egg development was done exactly as stated in *Method One*. The procedure was performed inside a water bath at 37.4°C± 0.2 to ensure that the eggs did not cool and that there was no reduction in blood flow [9]. The inner membranes were saturated with 0.9% NaCl and placed back into the incubator for no longer than 30 minutes. Next, the

NaCl solutions were decanted and the inner membranes were removed to expose the CAMs. The test was performed in the water bath instead of the incubator, as in *Method Two*, to avoid contact with the incubator fan. The fan was thought to contribute to the drying effect experienced by the eggs in *Method Two*.

One milliliter of both 0.9% NaCl, positive control, and 1% NaOH, negative control, were applied every 10 minutes for 1 hour.

Performed in triplicate, 0.5 ml of each test substance was applied to each CAM every 10 minutes for 1 hour instead of 1 ml, as used with the positive and negative controls. The one-milliliter volumes overloaded the CAMs and leaked over the sides of the eggs. Pictures were taken of the membrane before application, at 5 min, 10 min, 20 min, 30 min, 40 min, 50 min, and 60 min intervals with a D50 Nikon Digital Camera with DX Nikon zoom lens sitting atop a camera stand.

Oxidation runs, using free chlorine (NaOCl or $Ca(OCl)_2$) or ozone, were performed immediately after the oxidant spike was introduced to each sample. Concentrations of free chlorine and ozone were determined with HACH Method 80 for Free Chlorine and spectrophotometrically, respectively.

The CAMs were observed for hyperemia (expansion of vessels), vascular lysis (vessel disintegration), hemorrhage (bleeding from the vessels), protein coagulation, and/or blood coagulation [9].

Test material was classified as *Weakly Irritating* if only weak symptoms (e.g. hyperemia) were observed. Its classification was *Moderatey Irritating* if, in addition, single appearances of more severe irritations (e.g. hemorrhage, vascular lysis or

coagulation) were observed. Lastly, it was classified as *Severely Irritating* if more than one of these effects were observed [9].

Method Three was developed to continue investigating "Method Two HET-CAM Experimental Outline" (Table 3.2.). The test substances were still scored according to Erdinger et al.

4.RESULTS

4.1 METHOD ONE

The HET-CAM method showed no obvious signs of irritancy in the saltwater sources, no signs of irritancy in the same sources that had been oxidized with 3 mg/L of either chlorine or ozone, and no signs of irritancy with the 100 ppm chloroform or 200 ppm bromoform.

The following section shows before and after pictures of the chorionallantoic membranes (Figs. 4.1. through 4.19.), using the HET-CAM method. Some of the pictures show signs of coagulation, hemorrhaging, and/or vascular lysis, but this does not necessarily indicate irritancy. Preparation of the chorionallantoic membranes using *Method One* was very difficult. Removal of the ectodermal membranes frequently caused some trauma to the chorionallantoic membranes. Because this test is comparative, relating the chorionallantoic membrane's condition prior to the application of the test substance to its condition after the application of the test substance, and the lengthy development of the eggs (9 to 10 days), slightly damaged eggs were used.

Figure 4.1 shows the effects of 0.3 ml of 1% NaOH (positive control) applied to a slightly damaged CAM. Hemorrhaging was seen 3 seconds after application (7 "points"), vascular injection was seen 30 seconds after application (5 "points"), and coagulation was seen 2 minutes after application (7 "points"). The positive control received an IS of 19 – Strong Irritant.

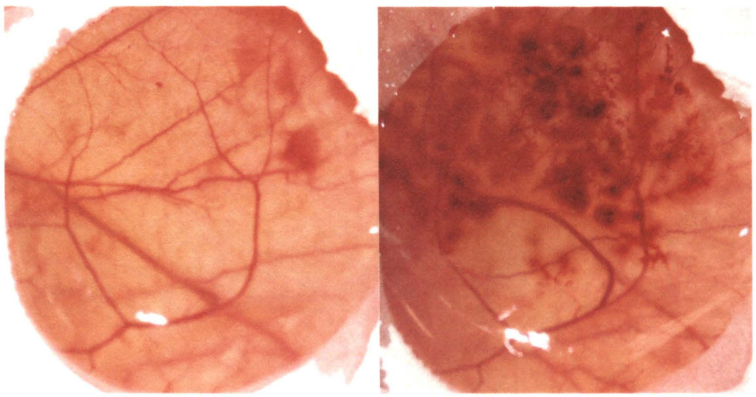

Figure 4.1. 1% NaOH positive control before application and 5 minutes after application.

Fig 4.2. shows the effects of 0.3 ml of 0.9% NaCl (negative control) applied to a CAM that was more than slightly damaged. No further hemorrhaging or other signs of irritancy were seen, other than that induced by preparation of the CAM. The change in shape of the hemorrhage was due to the application of the negative control.

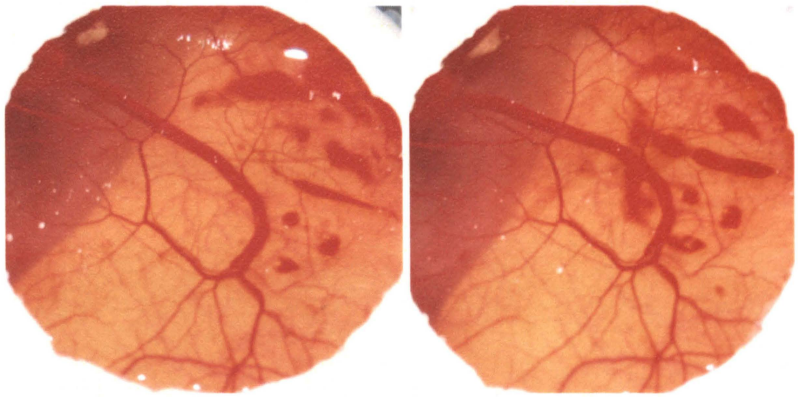

Figure 4.2. 0.9% NaCl negative control before application and 5 minutes after application.

Fig. 4.3 shows the effects of 0.3 ml of an OLMB sample applied to a slightly damaged CAM. Throughout, no obvious signs of irritation were observed.

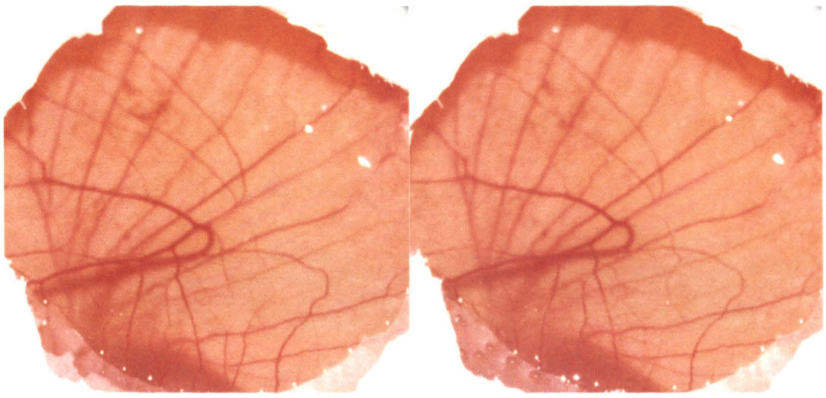

Figure 4.3. OLMB NO TREATMENT before application and 5 minutes after application.

Fig. 4.4 shows the effects of 0.3 ml of an OLMB sample, dosed with 3mg/L of free chlorine, applied immediately to a slightly damaged CAM. There was possibly a sign of vascular lysis (vessel disintegration) that appeared at 2 minutes (3 "points"). Three "points" were enough to be assessed as a "Slight Irritant." Because the effect was

so slight and the test was not performed in duplicate or triplicate, this test will not be conclusively classified as an ocular irritant.

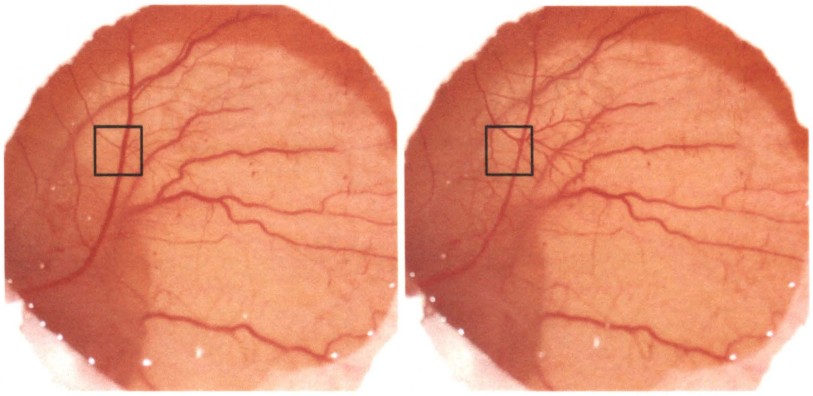

Figure 4.4. OLMB dosed with 3 mg/L of NaOCl as Cl_2 (1 minute disinfection time), before application and 5 minutes after application.

Fig. 4.5 shows the effects of 0.3 ml of an OLMB sample, dosed with 3 mg/L of free chlorine, applied to a slightly damaged CAM 30 min after dosage. Throughout, no obvious signs of irritation were observed.

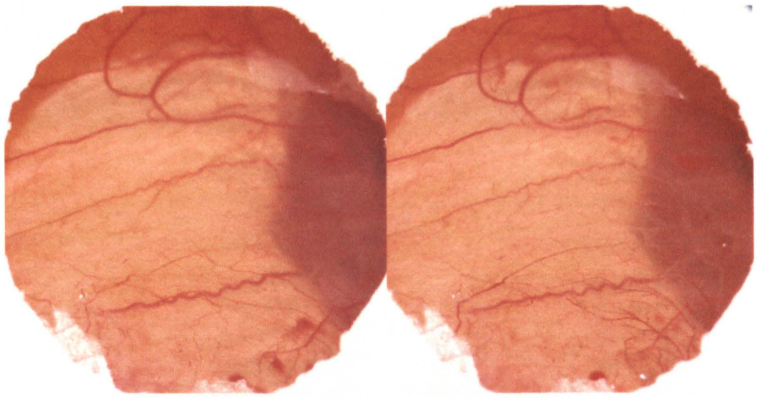

Figure 4.5. OLMB dosed with 3 mg/L of NaOCl as Cl_2 (30 minute disinfection time), before application and 5 minutes after application.

Fig. 4.6 shows the effects of 0.3 ml of an OLMB sample, dosed with 3 mg/L of O_3, applied to a slightly damaged CAM immediately. Throughout, no obvious signs of irritation were observed.

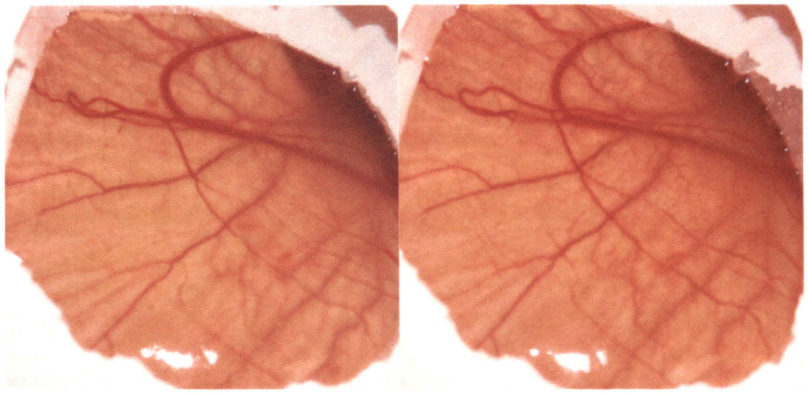

Figure 4.6. OLMB dosed with 3 mg/L of O_3 (1 minute disinfection time), before application and 5 minutes after application.

Fig. 4.7 shows the effects of 0.3 ml of a OLMB sample, dosed with 3 mg/L of O_3, applied to a slightly damaged CAM 30 min after dosage. Throughout, no obvious signs of irritation were observed.

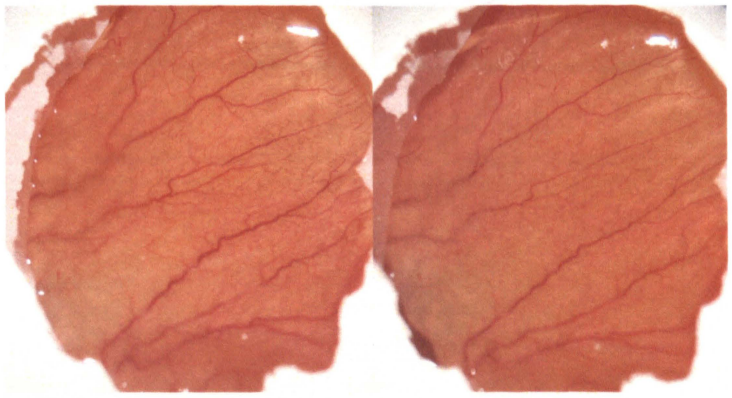

Figure 4.7. OLMB dosed with 3 mg/L of O_3 (30 minute disinfection time), before application and 5 minutes after application.

Fig. 4.8 shows the effects of 0.3 ml of a SDMB sample applied to a slightly damaged CAM. Throughout, no obvious signs of irritation were observed.

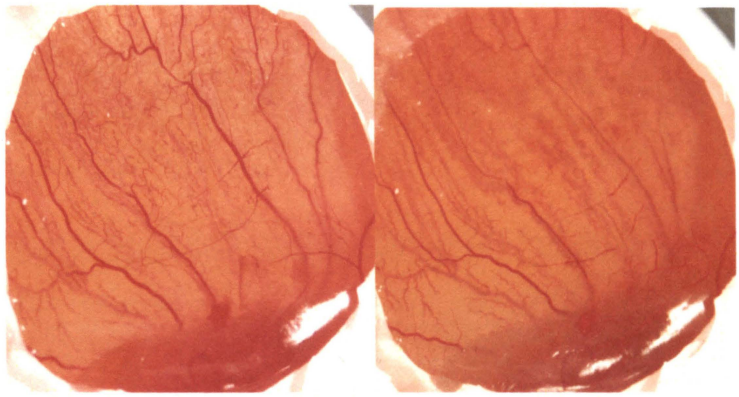

Figure 4.8 SDMB NO TREATMENT before application and 5 minutes after application.

Fig. 4.9 shows the effects of 0.3 ml of a SDMB sample, dosed with 3 mg/L of free chlorine, immediately applied to a slightly damaged CAM. Throughout, no obvious signs of irritation were observe.

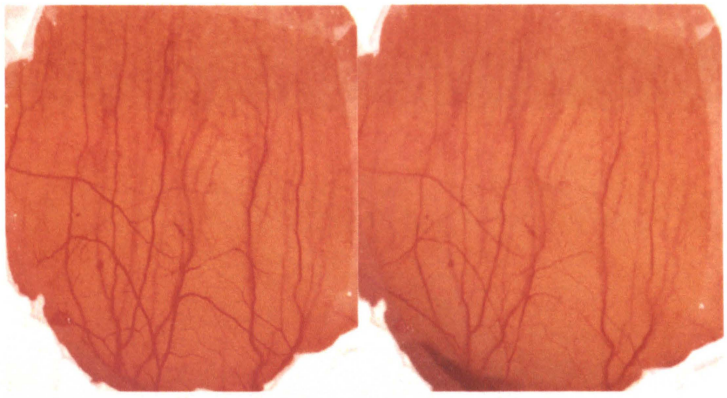

Figure 4.9. SDMB dosed with 3 mg/L of NaOCl as Cl_2 (1 minute disinfection time), before application and 5 minutes after application.

Fig. 4.10 shows the effects of 0.3 ml of a SDMB sample, dosed with 3 mg/L of free chlorine, applied to a slightly damaged CAM 30 min after dosage. Throughout, no obvious signs of irritation were observed.

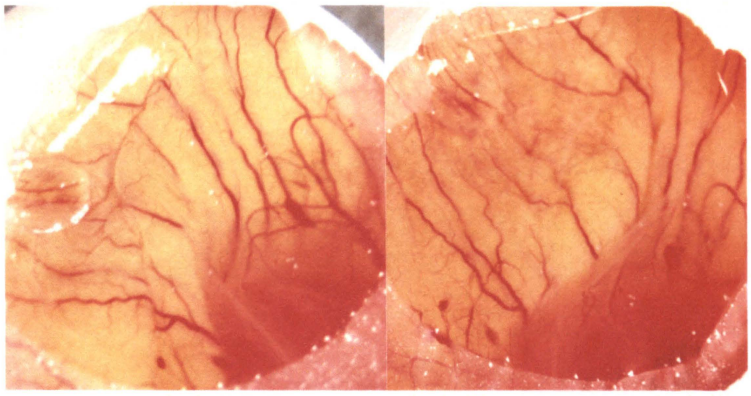

Figure 4.10. SDMB dosed with 3 mg/L of NaOCl as Cl_2 (30 minute disinfection time), before application and 5 minutes after application.

Fig. 4.11 shows the effects of 0.3 ml of a SDMB sample, dosed with 3 mg/L of O_3, was applied to a slightly damaged CAM immediately. Throughout, no obvious signs of irritation were observed.

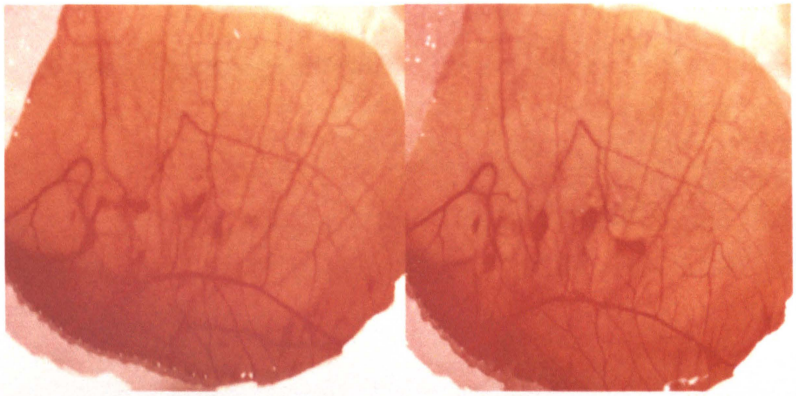

Figure 4.11. SDMB dosed with 3 mg/L of O_3 (1 minute disinfection time), before application and 5 minutes after application.

Fig. 4.12 shows the effects of 0.3 ml of a SDMB sample, dosed with 3 mg/L of O_3, applied to a well-prepared CAM 30 min after dose. Throughout, no obvious signs of irritation were observed.

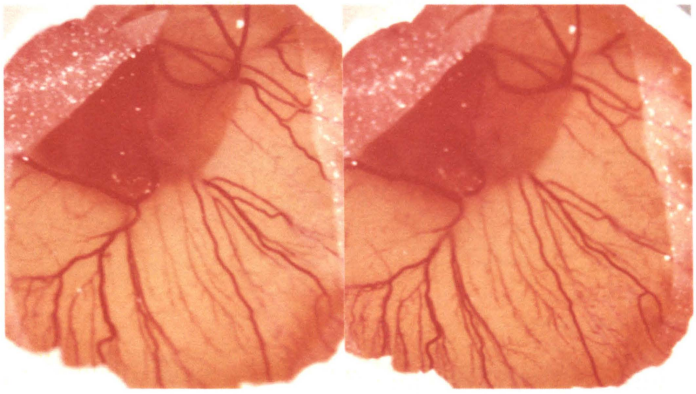

Figure 4.12. SDMB dosed with 3 mg/L of O_3 (30 minute disinfection time), before application and 5 minutes after application.

Fig. 13 shows the effects of 0.3 ml of a TXMB sample applied immediately to a slightly damaged CAM. Throughout, no obvious signs of irritation were observe. There was possibly a sign of vascular lysis (vessel disintegration) that appeared at 3 minutes (1 "point"). One "point" is enough to be assessed as a "Slight Irritant." Because the effect is so slight and the test was not performed in duplicate or triplicate, this test will not be conclusively classified as an ocular irritant.

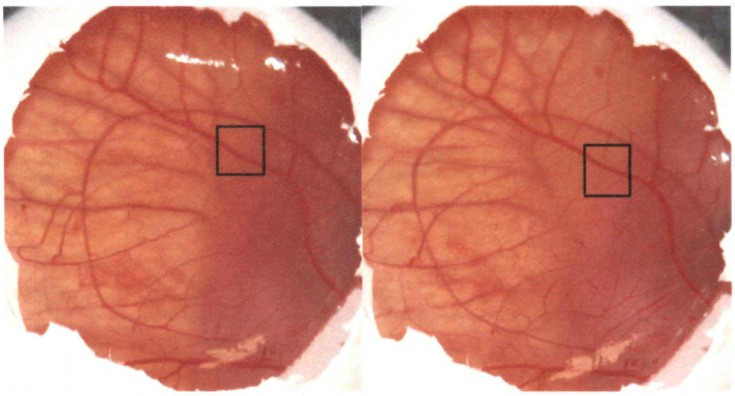

Figure 4.13. TXMB NO TREATMENT before application and 5 minutes after application.

Fig. 4.14 shows the effects of 0.3 ml of a TXMB sample, dosed with 3 mg/L of free chlorine, immediately applied to a slightly damaged CAM. Throughout, no obvious signs of irritation were observed.

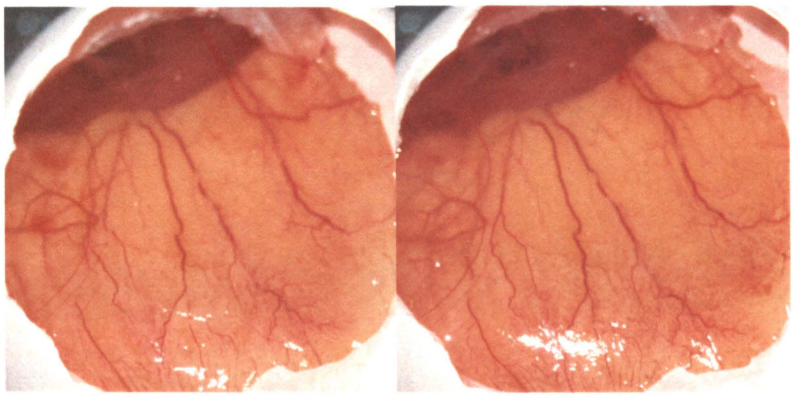

Figure 4.14. TXMB dosed with 3 mg/L of NaOCl as Cl_2 (1 minute disinfection time), before application and 5 minutes after application.

Fig 4.15 shows the effects of 0.3 ml of a TXMB sample, dosed with 3 mg/L of free chlorine, applied to a slightly damaged CAM 30 min after dosage. Throughout, no obvious signs of irritation were observed.

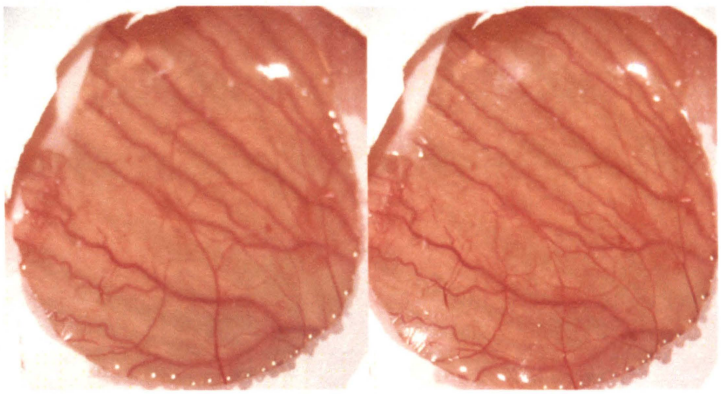

Figure 4.15. TXMB dosed with 3 mg/L of NaOCl as Cl_2 (30 minute disinfection time), before application and 5 minutes after application.

Fig. 1.6 shows the effects of 0.3 ml of a TXMB sample, dosed with 3 mg/L of O_3, immediately applied to a slightly damaged CAM. Throughout, no obvious signs of irritation were observed.

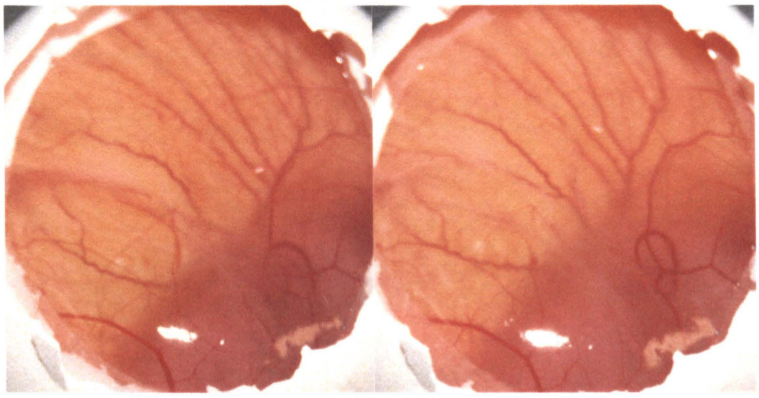

Figure 4.16. TXMB dosed with 3 mg/L of O_3 (1 minute disinfection time), before application and 5 minutes after application.

Fig. 14.7 shows the effects of 0.3 ml of a TXMB sample, dosed with 3 mg/L of O_3, applied to a slightly damaged 30 min after dosage. Throughout, no obvious signs of irritation were observed.

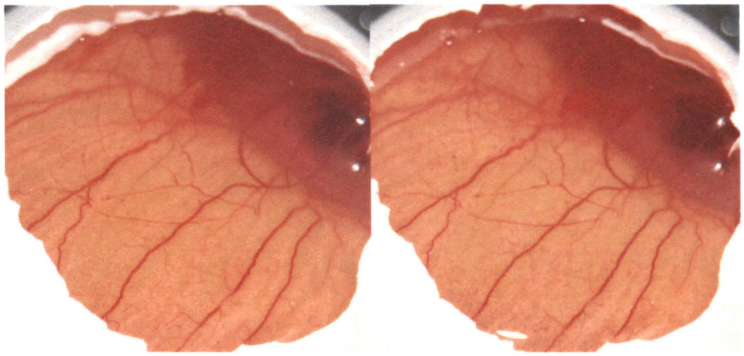

Figure 14.7. TXMB dosed with 3 mg/L of O_3 (30 minute disinfection time), before application and 5 minutes after application.

Fig. 14.8 shows the effects of 0.3 ml of 100 ppm of chloroform applied to a moderately damaged CAM. Throughout, no obvious signs of irritation were observed.

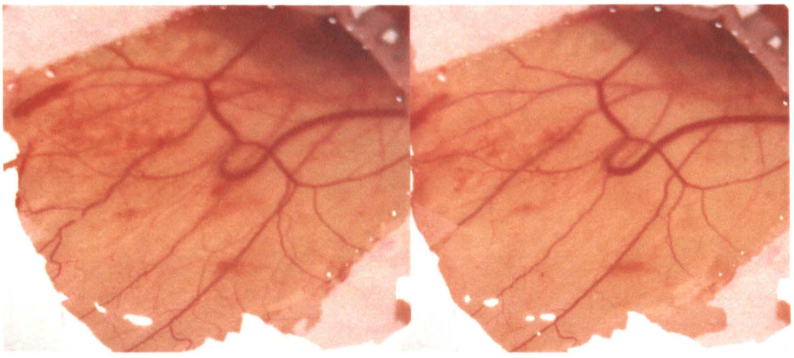

Figure 4.18. CHLOROFORM 100 ppm before application and 5 Minutes after application.

Fig. 4.19 shows the effects of 0.3 ml of 200 ppm of bromoform applied to a moderately damaged CAM. Throughout, no obvious signs of irritation were observed.

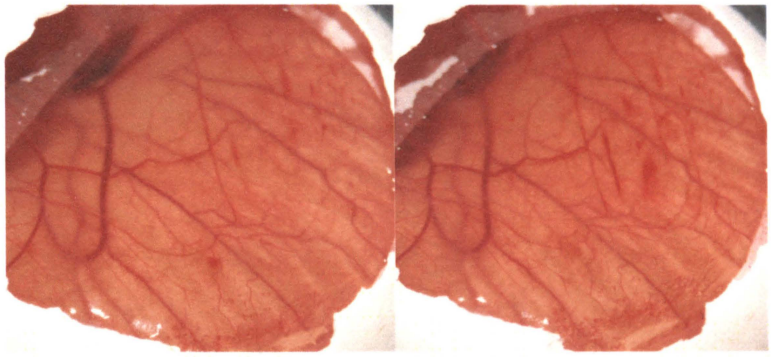

Figure 4.19. BROMOFORM 200 ppm before application and 5 Minutes after application.

4.2 METHOD TWO

Only a small fraction of "Method Two HET-CAM Experimental Outline" was attempted. The only samples tested were SDMB 10mg/L NaOCl as Cl_2, SDMB 100 mg/L NaOCl as Cl_2, and SDMB 1000 mg/L NaOCl as Cl_2. Reaction times between the saltwater sample and disinfectant were kept under 5 minutes. The method showed quite significant signs of irritancy.

The following section shows before and after pictures (Figs. 4.20. through 4.22.) of the CAMs using the HET-CAM method. Only one picture of the eggs used in the triplicate test is provided. Preparation with *Method Two* proved to be much easier than with *Method One* because it significantly reduced the trauma caused to the CAMs during removal of the ectodermal membranes.

Fig. 4.20 shows the effects of 0.3 ml of a SDMB sample, dosed with 10 mg/L of free chlorine, applied to a well-prepared CAM and observed for 1 hour. Though difficult

to see, irritancy was present. The CAM, along with the vessels, appeared to have dried out and hardened slightly, a consequence of the coagulation of blood within the vessels and the protein surrounding them. Slight hyperemia was also noticeable. Because coagulation of blood and protein, as well as hyperemia, was occurring, "SDMB 10mg/L Free Chlorine" seemed to be *Severely Irritating*.

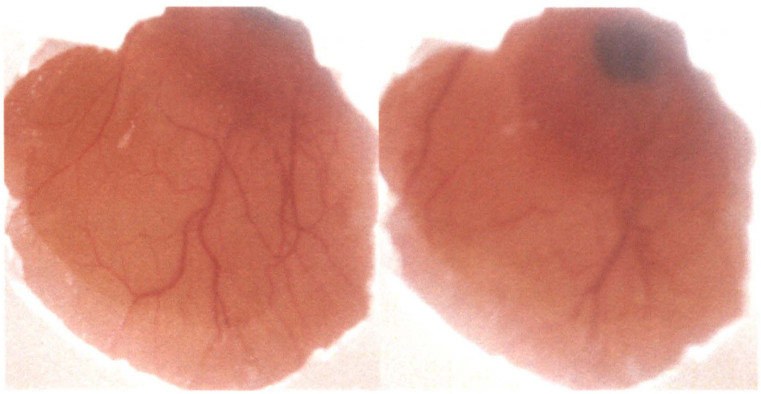

Figure 4.20. SDMB dosed with 10 mg/L NaOCl as Cl_2, before application and 1 hour after application.

Fig. 4.21 shows the effects of 0.3 ml of a SDMB sample, dosed with 100 mg/L of free chlorine, applied to a well-prepared CAM and observed for 1 hour. Though difficult to see, irritancy was present. The CAM, along with the vessels, appeared to have dried out and hardened slightly, a consequence of the coagulation of blood within the vessels and the protein surrounding them. Slight hyperemia was also noticeable. Because coagulation of blood and protein, as well as hyperemia, was occurring, "SDMB 100 mg/L Free Chlorine" seemed to be *Severely Irritating*.

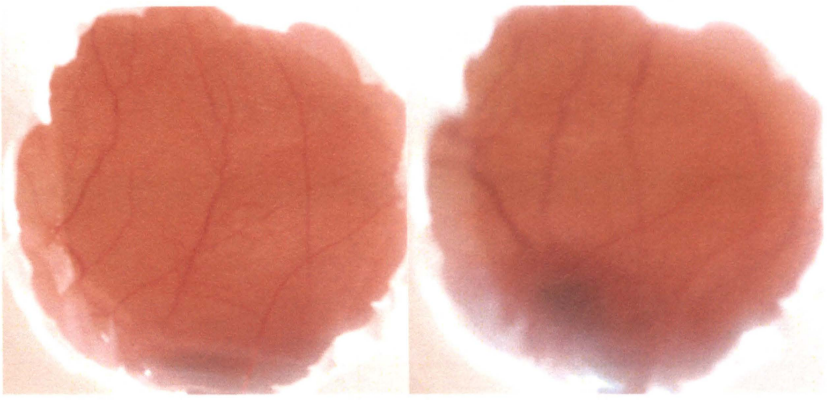

Figure 4.21. SDMB dosed with 100 mg/L NaOCl as Cl_2, before application and 1 hour after application.

Fig. 22 shows the effects of 0.3 ml of a SDMB sample, dosed with 1000 mg/L of free chlorine, applied to a well-prepared CAM and observed for 1 hour. There was a strong presence of irritancy. The CAM, along with the vessels, appeared to have dried out and hardened slightly, a consequence of the coagulation of blood within the vessels and the protein surrounding them. Slight hyperemia was also noticeable. Because coagulation of blood and protein, as well as hyperemia, was occurring, "SDMB 100 mg/L Free Chlorine" seemed to be *Severely Irritating*.

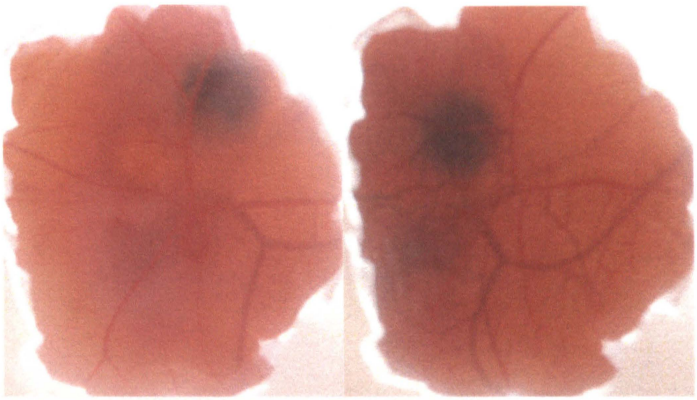

Figure 4.22. SDMB dosed with 1000 mg/L NaOCl as Cl_2, before application and 1 hour after application.

4.3 METHOD THREE

When using *Method Three* (a slight alteration to *Method Two*), only a small fraction of "Method Two HET-CAM Experimental Outline" was accomplished. Samples tested were 0.9% NaCl Negative Control, 1% NaOH Positive Control, SDMB dosed with 10 mg/L NaOCl as Cl_2, SDMB dosed with 100 mg/L NaOCl as Cl_2, SDMB dosed with 10 mg/L $Ca(OCl)_2$ as Cl_2, and SDMB dosed with 100 mg/L $Ca(OCl)_2$ as Cl_2. Reaction times between the saltwater sample and disinfectant were kept under 5 minutes. The method showed signs of irritancy.

Also, Erdinger et al.'s synergistic affect was investigated. Samples tested were SDMB no treatment, SDMB dosed with 3 mg/L NaOCl as Cl_2, SDMB, dosed with 0.1 mg bromoform and 3 mg/L NaOCl as Cl_2, and SDMB dosed with 1 mg bromoform and 3 mg/L NaOCl as Cl_2.

The following section shows before and after pictures of the CAMs using the HET-CAM method (Figs. 4.23. through 4.32.). Only one egg, two pictures, used in the triplicate test is provided. Preparation with *Method Two* proved to be much easier than with *Method One*, significantly reducing the trauma caused to the CAM during the removal of the ectodermal membrane.

Fig. 4.23. shows the effects of 1ml of 0.9% NaCl (negative control) applied to a well-prepared CAM every 10 minutes. Throughout, no signs of irritancy were observed.

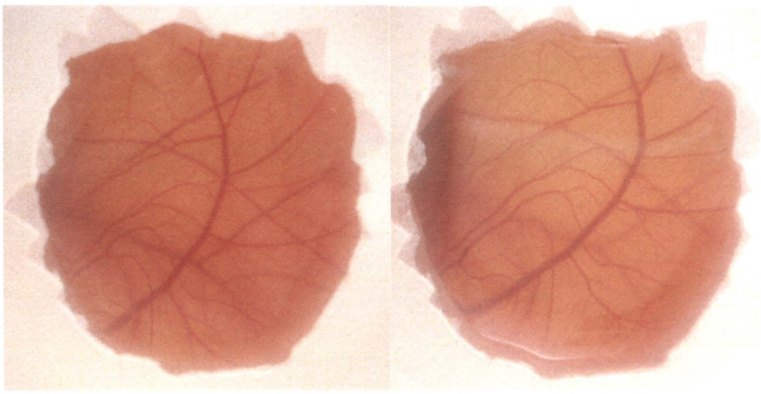

Figure 4.23. 0.9% NaCl negative control before application and 1 hour after application.

Fig. 4.24 shows the effects of 1ml of 1% NaOH (positive control) applied to a well-prepared CAM every 10 minutes. Hemorrhaging was seen 3 seconds after application of positive control, and coagulation of blood and protein were also observed. The positive control was considered *Severely Irritating.*

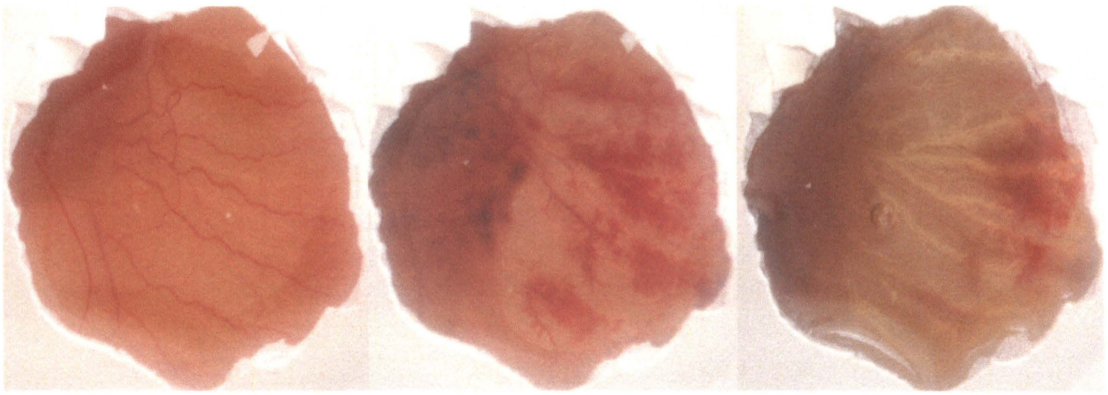

Figure 4.24. 1% NaOH positive control before application, 5 minutes after application, and 1 hour after application.

Fig. 4.25 shows the effects of 0.5 ml of the test substance added to a well-prepared CAM every 10 minutes. In general, all CAMs experienced slight drying and vascular lysis, and two experienced very slight hemorrhaging. Although, according to the Erdinger et al. scoring system, the test substance that caused reactions in two eggs should be considered *Moderately Irritating* and the other *Weakly Irritating*, the irritation effects were so minor that the test substance was subjectively determined to be only *Weakly Irritating*.

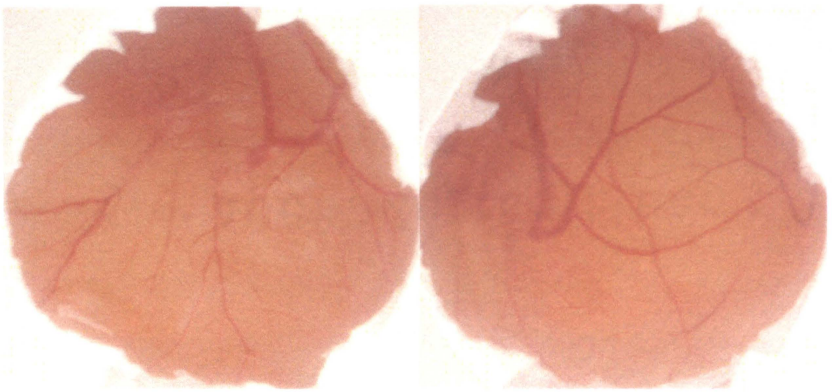

Figure 4.25. SDMB dosed with10 mg/L NaOCl as Cl_2, before application and 1 hour after application.

Fig. 4.26 shows the effects of 0.5 ml of the test substance added every to a well-prepared CAM 10 minutes. One CAM underwent slight drying out and slight protein coagulation, the next underwent significant protein coagulation and slight hemorrhaging, and the last underwent moderate hemorrhaging and moderate protein coagulation. According to the Erdinger et al. scoring system, the test substance was classified as *Moderately Irritating*.

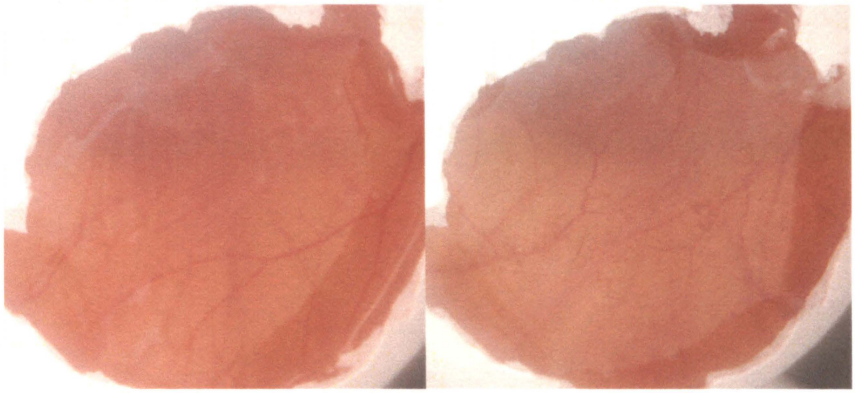

Figure 4.26. SDMB dosed with 100 mg/L NaOCl as Cl_2, before application and 1 hour after application.

Fig. 4.27 shows the effects of 0.5 ml of the test substance was added to a well-prepared CAM every 10 minutes. In general, all CAMs experienced slight protein coagulation, and one CAM, given above, underwent vascular lysis. Though according to the Erdinger et al. scoring system, the test substance that caused reactions in two eggs should be considered *Weakly Irritating* and the other *Moderately Irritating*, the irritation effects were so minor that the test substance was subjectively determined to be only *Weakly Irritating*.

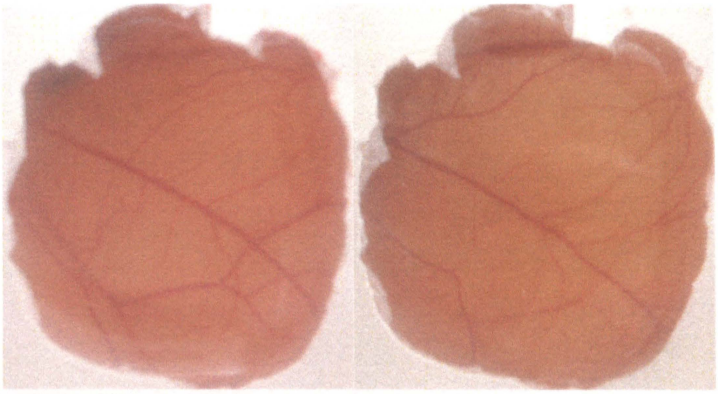

Figure 4.27. SDMB dosed with 10 mg/L $Ca(OCl)_2$ as Cl_2, before application and 1 hour after application.

Fig. 4.28. shows the effects of 0.5 ml of the test substance added to a well-prepared CAM every 10 minutes. In general, all CAMs experienced slight to moderate hemorrhaging and vascular lysis. According to the Erdinger et al. scoring system, the test substance was classified as *Moderately Irritating*

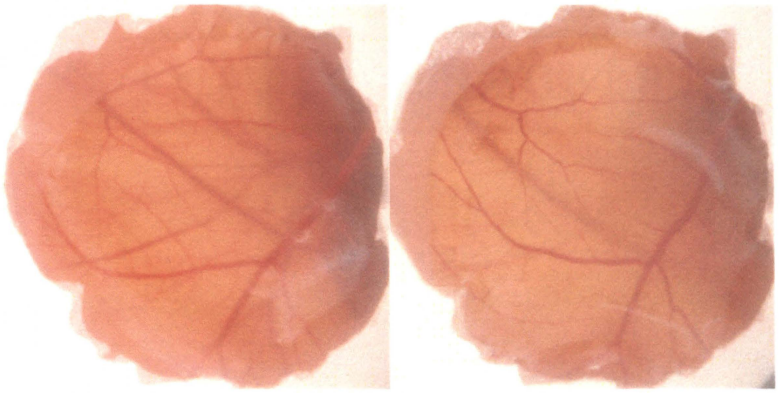

Figure 4.28. SDMB dosed with100 mg/L $Ca(OCl)_2$ as Cl_2, before application and 1 hour after application.

Fig. 4.29. shows the effects of 0.5 ml of the test substance added to a well-prepared CAM every 10 minutes. In general, all CAMs experienced moderate hemorrhaging, vascular lysis, and coagulation of blood. According to the Erdinger et al. scoring system, the test substance was classified as *Severely Irritating*.

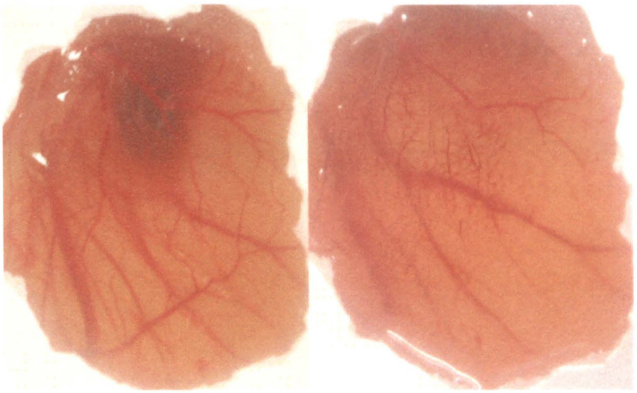

Figure 4.29. SDMB no treatment before application and 1 hour after application.

Fig. 4.30. shows the effects of 0.5 ml of the test substance added to a well-prepared CAM every 10 minutes. In general, all CAMs experienced slight hemorrhaging and slight vascular lysis. According to the Erdinger et al. scoring system, the test substance was classified as *Weakly Irritating*.

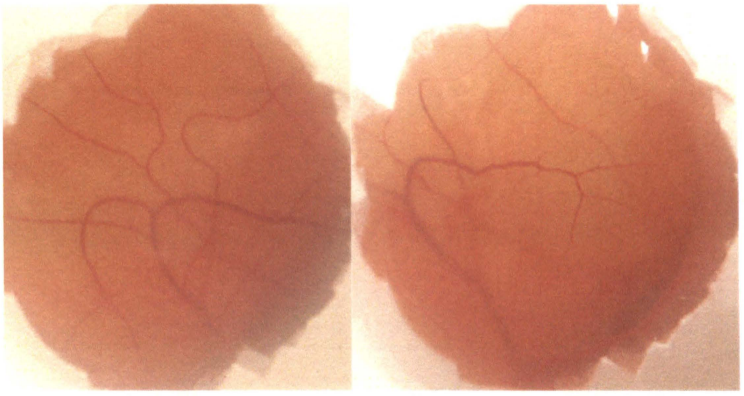

Figure 4.30. SDMB dosed with 3 mg/L NaOCl as Cl_2, before application and 1hour after application.

Fig. 4.31. shows the effects of 0.5 ml of the test substance added to a well-prepared CAM every 10 minutes. All CAMs experienced slight to moderate

hemorrhaging, and two eggs, further, experienced slight vascular lysis and slight coagulation of blood. According to the Erdinger et al. scoring system, the test substance was classified as *Moderately Irritating*.

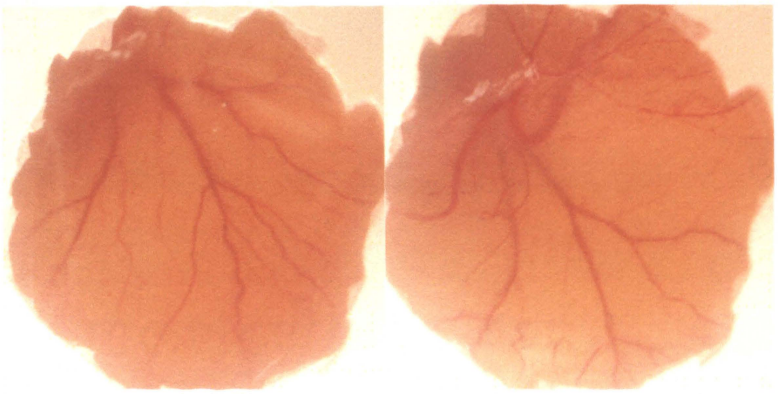

Figure 4.31. SDMB dosed with 0.1 mg/L bromoform and 3 mg/L NaOCl as Cl_2, before application and 1hour after application.

Fig. 4.32. shows the effects of 0.5 ml of the test substance added to a well-prepared CAM every 10 minutes. In general, all CAMs experienced slight to moderate hemorrhaging, vascular lysis and coagulation of blood. According to the Erdinger et al. scoring system, the test substance was classified as *Severely Irritating*.

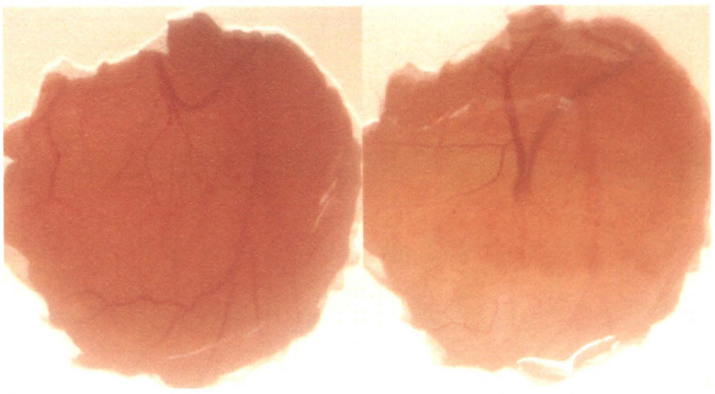

Figure 4.32. SDMB dosed with 1 mg/L bromoform and 3 mg/L NaOCl as Cl_2, before application and 1hour after application.

5. DISCUSSION

5.1. METHOD ONE

Preparation of the CAM in *Method One* proved to be extremely difficult, so a new method had to be developed. Spielmann replaced 0.9% NaCl saturated ectodermal membranes in an incubator for no more than 30 minutes. This softened the ectodermal membrane significantly allowing it to be separated from CAM with greater ease, less damage, and more reproducibility. Also, the entire test was performed in an incubator allowing for the extension of the observation duration to 1 hour. By keeping the eggs in an incubator for the duration of the test, it ensured that the eggs were at developmental specifications. Because developmental conditions were maintained, there was no cooling of the eggs hence any reduction in blood flow [9].

5.2. METHOD TWO

Method Two, in theory, allowed the observation duration to be extended to 1 hour since eggs were kept in an incubator at developmental specifications. Keeping the eggs at developmental specifications helped to ensure that they did not cool and that there was no reduction in blood flow [9]. The move brought unforeseen factors. Drying out of the CAM appeared to be one of the major ocular effects seen during the test. The problem then became whether or not the extended duration in the incubator was the culprit, or if the test substances were solely at fault. Unfortunately, at this point in the investigation, method development was such a priority that not all test substances within an experimental matrix were considered. Had a positive and/or negative control been

investigated, this question may have been answered. The incubator housed small fans that distributed heat evenly, and the question arose as to whether or not these fans had a part in the "drying out" of the CAM.

Erdinger et al. performed their test in an incubator, but also had a constant application of the test substance (1.25 ml/min). This suggested that they possibly also saw a "drying out" of the CAM during the 1-hour extended duration. In *Method Three*, the test was moved from an incubator to a water bath, and 0.5 ml of each test substance was added every 10 minutes for 1 hour.

5.3. METHOD THREE

Method Three proved to be the best method used to date in the experiment. The combination of both performing the experiment in a water bath (as opposed to the incubator, as done in *Method Two*) and adding 0.5 ml of a test substance every 10 minutes for 1 hour helped to extend the observation duration while reducing possible environmental effects to the CAM. Such problematic effects included, but are not limited to, the possibility that the incubator fan played a part in the "drying out" of the CAM.

5.4. DEVELOPMENTAL PROBLEMS

Viable egg development was a problem throughout the entire experiment. As indicated by several of the sources cited, the age of the eggs, temperature, humidity, and rotation are key components to the proper development of the eggs. Another factor *NOT* mentioned in these sources is the disinfection and cleanliness of the incubator used. Following is a discussion of each of these factors.

5.4.1 Egg Age. As stated in several sources, eggs must be no older than 7 days before introduction into an incubator. One problem faced by investigators is the location of their egg suppliers, as well as the means by which the suppliers obtain their eggs.

Moyer's Chicks, Quakertown, PA, provided eggs used in this experiment. Eggs were always well packed and very few cracked eggs were received, but the age of the eggs was not always known. Moyer's Chicks obtain its fertile white Leghorn chicks from another supplier.

When finding an egg supplier, it is recommended that one be selected that is close to your lab, and, also, determine whether that supplier owns the hatchery or obtains the eggs from another supplier.

5.4.2 Temperature/Humidity. Temperature is critical in egg development. Early in the investigation, eggs were received in bulk. Those not initially placed in the incubator were put into a refrigerator. The temperature and humidity in the incubator were allowed to become steady before any eggs were introduced. The temperature of the eggs themselves was required to come to room temperature before being placed in the incubator.

In the winter months, viability was greatly reduced. Though this may have been accounted for by the cleanliness of the incubator (which will be discussed later), it is something to consider. During the winter months, eggs will undergo multiple temperature changes, often drastic, during delivery. The journey from a warm hatchery, to a cold delivery truck, to a possibly warm shipping station, to a cold delivery truck, to a warm lab *may* adversely affect the proper development of the eggs.

5.4.3 Rotation. An automatic turner provided by Brinsea, Inc. rotated the eggs in the lab, which took the place of hand rotation, as suggested by the NICEATM-ICCVAM Report (2006).

Once during the investigation, the incubator was accidentally placed on an unplugged automatic turn cradle. After 9 days, none of the eggs had developed, which suggested, as was already known, that rotation of eggs is crucial.

5.4.4 Disinfection/Cleanliness. Unstated in all of the sources cited, is the importance of disinfection and cleansing of the incubator. As egg viability became of increasing concern, and each the above factors was checked and confirmed, it was suggested by Brinsea, Inc. that the incubator might to be need disinfected. If egg development continues in incubators without cleansing and disinfection, bacteria can grow, eventually penetrating the porous shells, and infecting the embryos, greatly reducing the number of viable eggs [10].

Commercially, disinfection is often done with a combination of the oxidizing agent potassium permanganate and formalin (40% formaldehyde). Formaldehyde, the toxic gas produced, is easy to use and an effective disinfectant [10].

It was suggested to use 42ml of formalin plus 21g of potassium permanganate per m^3 for 20 to 40 minutes in the hood [10]. Estimating that the incubator in use was under $0.3m^3$, the amounts were adjusted, as necessary, and allowed to fumigate for 30 minutes under the hood.

6. CONCLUSIONS

6.1. METHOD ONE

There were two appearances of *possible* irritation: "TXMB NO TREATMENT before application and 5 minutes after application" and "OLMB 3 mg/L of NaOCl as Cl_2 (1 minute disinfection time) before application and 5 minutes after application." Because the effect were so slight, the tests were not performed in duplicate or triplicate, and no other irritations were seen within their respective matrices, the two samples were NOT conclusively considered ocular irritants and were labeled with asterisks (Table 6.1).

Though no *obvious* signs of ocular irritation were observed using this method, these results were not conclusively indicative of those solutions that are not ocular irritants. These solutions were either not ocular irritants or were at concentrations below the sensitivity of the test. Remember, the HET-CAM has not been validated to distinguish between ocular irritants and non-ocular irritants.

6.2. METHOD TWO

Signs of ocular irritancy were present in all three tests performed in triplicate (SDMB 10 mg/L NaOCl as Cl_2, SDMB 100 mg/L NaOCl as Cl_2, and SDMB 1000 mg/L NaOCl as Cl_2). Unfortunately, the camera was out of focus during the procedure and the irritancy was difficult to recall visually. Also, remember that in this method, the scoring guide developed by Luepke could not be continued because the duration of the investigation was extended to 1 hour.

Table 6.1. Method One HET-CAM Experimental Outline with IS SCORES

Run	Sample[a]	Oxidant[b]	Oxidant Dose (mg/L)	Time after Oxidant Spike (min)	Note	IS SCORE
1	+ CONTROL	NA	NA	NA	1%NaOH	17
2	- CONTROL	NA	NA	NA	0.9%NaCl	0
3	OLMB	NA	NA	NA	CONTROL	0
4	OLMB	Cl_2	3	1		3
5	OLMB	Cl_2	3	30		0
6	OLMB	O_3	3	1		0
7	OLMB	O_3	3	30		0
8	SDMB	NA	NA	NA	CONTROL	0
9	SDMB	Cl_2	3	1		0
10	SDMB	Cl_2	3	30		0
11	SDMB	O_3	3	1		0
12	SDMB	O_3	3	30		0
13	TXMB	NA	NA	NA	CONTROL	1
14	TXMB	Cl_2	3	1		0
15	TXMB	Cl_2	3	30		0
16	TXMB	O_3	3	1		0
17	TXMB	O_3	3	30		0
18	chloroform	NA	NA	NA	100ppm	0
19	bromoform	NA	NA	NA	200ppm	0

By using the subjective classification scheme as developed by Erlinger et al., "SDMB 10 mg/L NaOCl as Cl_2," "SDMB 100 mg/L NaOCl as Cl_2," and "SDMB 1000 mg/L NaOCl as Cl_2" were all classified as *Severely Irritating*. The blood vessels appeared to have expanded (hyperemia) and the CAMs, along with the vessels, appeared to have dried and hardened slightly, a consequence of coagulation of the blood within the vessels and the protein surrounding them (Table 6.2).

Table 6.2. Method Two HET-CAM Experiment Outline with Irritation Classifications

	Sample[a]	Oxidant	Dose mg/L as Cl_2	Classification
1	SDMB	NaOCl	10	*Moderately*
2		NaOCl	100	*Severely*
3		NaOCl	1000	*Severely*
4	SDMB	$Ca(OCl)_2$	10	
5		$Ca(OCl)_2$	100	
6		$Ca(OCl)_2$	1000	

	Sample	Oxidant	Dose	Classification
7	SDMB	O_3	10	
8		O_3	100	
9		O_3	1000	

Only part of Table 3.2 is shown with irritation classifications because only a few were completed.

6.3. METHOD THREE

6.3.1 Method Three. Signs of ocular irritancy were present in all four tests (Table 6.3), performed in triplicate (SDMB 10 mg/L NaOCl as Cl_2, SDMB 100 mg/L NaOCl as Cl_2, SDMB 10 mg/L $Ca(OCl)_2$ as Cl_2, and SDMB 100 mg/L $Ca(OCl)_2$ as Cl_2). Unfortunately, the camera did not remain focused and results were difficult to recall visually. Once again, the scoring guide developed by Luepke could not be continued because the duration of the investigation was extended to 1 hour.

"SDMB 10 mg/L NaOCl as Cl_2" caused slight drying and vascular lysis to all CAMs, while two experienced very slight hemorrhaging. One occurrence of hemorrhage appeared to be typical with small dendrites of blood from vessel branches, while the other was a very small spot of blood. The small spot of blood was not typical, and it is thought

that the vessel may have been damaged during preparation; the small blood spot appeared early, at 5 minutes. Based on the subjective classification scheme, as developed by Erlinger et al., and considering that the hemorrhaging was so slight, "SDMB 10 mg/L NaOCl as Cl_2" was classified as *Weakly Irritating*.

"SDMB 100 mg/L NaOCl as Cl_2" caused significant protein coagulation, slight to moderate hemorrhaging, and slight to moderate protein coagulation among the three CAMs. Based on the subjective classification scheme of Erlinger et al., "SDMB 10 mg/L NaOCl as Cl_2" was classified as *Moderately Irritating*.

"SDMB 10 mg/L $Ca(OCl)_2$ as Cl_2" caused slight protein coagulation, and one CAM underwent vascular lysis. Based on the subjective classification scheme of Erlinger et al., the test substance that caused reactions in two eggs should be considered *Weakly Irritating* and the other *Moderately Irritating*. Because these ocular irritation effects were so minor, "SDMB 10 mg/L $Ca(OCl)_2$ as Cl_2" was classified as *Weakly Irritating*.

"SDMB 100 mg/L $Ca(OCl)_2$ as Cl_2" caused Weak to moderate hemorrhaging and vascular lysis among all CAMs. Based on the subjective classifications scheme of Erlinger et al., "SDMB 100 mg/L $Ca(OCl)_2$ as Cl_2" was classified as *Moderately Irritating*.

Though both disinfectants, NaOCl and $Ca(OCl)_2$, caused slightly and moderately irritating effects, in general, the effects of $Ca(OCl)_2$ were milder than those of NaOCl.

Table 6.3. Method Two HET-CAM Experimental Outline (Using Method Three) with Irritation Classifications

	Sample[a]	Oxidant	Dose mg/L as Cl_2	Classification
1	SDMB	NaOCl	10	*Weakly*
2		NaOCl	100	*Moderately*
3		NaOCl	1000	
4	SDMB	$Ca(OCl)_2$	10	*Weakly*
5		$Ca(OCl)_2$	100	*Moderately*
6		$Ca(OCl)_2$	1000	

	Sample	Oxidant	Dose	Classification
7	SDMB	O_3	10	
8		O_3	100	
9		O_3	1000	

Only part of Table 3.2 is shown with irritation classifications because only a few were completed.

6.3.2 Method Three: Synergistic Effect. The results of the synergistic matrix were interesting (Table 6.4). The "SDMB no treatment" test solution was classified, according to Erdinger et al.'s scoring system, as *Severely Irritating* because of its moderate hemorrhaging, vascular lysis, and coagulation of blood. (The *Severely Irritating* classification of "SDMB no treatment" test solution is surprising, and the explanation is unknown.) The "SDMB dosed with 3 mg/L NaOCl as Cl_2" test solution was classified as *Weakly Irritating* because of its slight hemorrhaging and slight vascular injection. The "SDMB dosed with 0.1 mg/L bromoform and 3 mg/L NaOCl as Cl_2" test solution was classified as *Moderately Irritating* because all CAMs experienced slight to moderate hemorrhaging, but only two CAMs experienced slight vascular lysis and slight coagulation. The "SDMB dosed with 1mg/L bromoform and 3 mg/L NaOCl as Cl_2" test

solution was classified as *Severely Irritating* because all CAMs experienced slight to moderate hemorrhaging, vascular lysis, and coagulation of blood.

It should be noted that there was no "SDMB dosed with 0.1 mg/L bromoform" or "SDMB dosed with 1 mg/L bromoform." These samples should have been run to complete the experimental matrix but there were not enough eggs.

Table 6.4. Method Three Synergistic Effects Experimental Results

	Sample[a]	Oxidant	Dose mg/L as Cl_2	Synergistic Addition	Dose mg/L	Classification
1	SDMB	NA	NA	NA	NA	*Severely*
2		NaOCl	3	NA	NA	*Weakly*
3		NaOCl	3	Bromoform	0.1	*Moderately*
4		NaOCl	3	Bromoform	1	*Moderately*

Though the synergistic matrix had some surprises, the synergistic effect was observed. The combination of free chlorine and bromoform did increase the irritancy of the solution. The results of this synergistic matrix are indicative of Erdiner et al.'s statement that the synergistic effect between chlorine and some halogen-containing organic compounds lowers the ocular irritation threshold of these compounds into the range of concentrations found in the water of swimming pools [9].

6.4 CONTINUING INVESTIGATION

The time required developing a working method left little time to obtain pertinent results. The results at hand do, however, serve as a guideline and head start for anyone who may wish to pursue this project/method further.

Further investigations of the HET-CAM would include completing the "Method Two HET-CAM Experimental Outline" using *Method Three*. Once that outline is complete, the concentration range of each disinfectant inducing damage to the CAM (inducing an ocular irritation effect) would be refined. This would give investigators a working disinfection concentration range of ocular irritation.

The next set of experiments would look at ocular irritation induced by disinfection byproducts through a range of concentrations. Each disinfection byproduct found in the site samples would be prepared at different concentrations in hopes that an ocular irritation threshold concentration (or concentration range) could be determined.

Finally, the synergistic effect would be pursued testing the different chemical classes/disinfection byproducts found in the saltwater samples of the aquatic theme parks (e.g. haloamines, haloacetic acids, halomethanes, and halonitromethanes). The matrix would test how low the concentrations of the compounds could be in order to induce irritation. This set of experiments would be rather cumbersome and extensive, but could reveal valuable insight to ocular irritancy in seawater/salt water sources.

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