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SOAPS AND DETERGENTS:

IN VITRO EYE IRRITATION TESTS

APRIL 4, 1994

PREPARED BY:

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Introduction

For almost 50 years the Draize eye irritation test (Draize, 1944) has been the accepted standard for substantiating the safety of consumer products, ocular medications and chemicals that might accidentally be splashed into the eyes of workers (McCulley and Stephens, 1993). The purpose of this rabbit eye test was to assess the potential of a chemical to cause ocular irritation to predict the effect such chemicals might have on the human eye.

Although the Draize eye irritation test can clearly identify substances that are highly irritative or completely innocuous, a number of problems are intrinsic to the test. One is that the rabbit eye differs from the human eye in that it has a thinner cornea, a nictitating membrane (third eyelid), a different tearing mechanism and a higher pH of the aqueous humor; thus, the rabbit eye is usually more sensitive to chemical insult than the human eye. Furthermore, the relevance of the test to human hazard evaluation is questionable because most testing protocols require application of the chemical into the lower everted eyelid and subsequent closure of the eyelids for 1-2 seconds. Such exposure is not representative of the typical human exposure to eye irritants (Talsma et al., 1988). Finally, the Draize eye irritation test is costly in terms of time and resources, uses significant numbers of animals and may be painful to the test animals.

During the '80s, animal rights organizations focused their attention on the Draize eye irritation test in their efforts to eliminate all safety testing in live animals. Government regulatory agencies and industrial corporations in the health-care field responded by seeking to develop alternative methods for testing for ocular toxicity. Primary dermal irritation tests are not reliable substitutes for in vivo ocular irritation assays (Gilman et al., 1983). Talsma et al. (1988) reported that highly accurate testing could be performed with fewer animals than the required six, and that sample size could be reduced (from the required six) to three and still preserve very good accuracy. Topical ophthalmic anaesthetics were proposed to decrease pain in test animals, but their use was not found to be entirely satisfactory (Seabaugh et al., 1993). Decreasing the quantity of test material ten fold (from 0.1 ml required in the Draize eye irritation procedure) has also become an acceptable modification (Lambert et al., 1993). Studies with 22 test materials conducted by the Soap and Detergent Association (Bagley et al., in press) have shown that a 93% correlation exists between results from this Low Volume Eye Test (LVET) and the Draize eye irritation test. Other in vivo testing procedures have included examining the corneal thickness (Kennah et al., 1989) and evaluating the corneal nerve irritation (Beuerman et al., 1992a). However, the major focus of the research was the replacement of the Draize eye irritation test with a suitable in vitro test.

Numerous in vitro tests have been advocated and developed over the last ten years. Some of these tests appear to have a great deal of promise; others have many drawbacks and still others are somewhere in between. An excellent overview of the various tests can be gained by reading the following recent comprehensive reviews: "Evaluation of Alternatives to the Draize Eye Test"

Table 1: *In Vitro* Test Systems and References

TEST SYSTEMS	REFERENCES
Agarose Diffusion	Bernhofer et al.(1993); Goldberg and Silber (1992); Rougier et al. (1992)
Chorioallantoic Membrane (CAM; HET-CAM; CAM/VA)	Goldberg and Silber (1992); Spielman et al. (1993); Bruner et al. (1991a); Rougier et al. (1992); Bagley et al. (in press); PMA (1990)
Enucleated Eye Test BEET (Bovine); CEET (Chicken); REET (Rabbit); PEET (Porcine)	PMA (1990); Price and Andrews (1985); Igarashi and Northover (1987); Burton et al. (1981); Sivak et al. (1992); Muir (1984, 1985); Rougier et al. (1992); Gautheron et al. (1992); Vanparys et al. (1993); Bruner et al. (1991a); Prinsen (1993); Prinsen and Koetner (1993); Berry and Easty (1993); Nuyts et al. (1990); Stankowski et al. (1993)
EYTEX (Protein Matrix Assay)	Rowan and Andrutis (1989); Goldberg and Silber (1992); Gordon (1992); PMA (1990); Decker and Harper (1993); Rougier et al (1992); McCulley and Stephens (1993); Gettings (1993); Bruner et al. (1991a)
Invertebrate Models Microtox/Luminescent Bacteria	Bruner et al (1991a); Rougier et al. (1992); Miller et al. (1993); McCulley and Stephens (1993); Goldberg and Silber (1992)
Tetrahymena Motility	PMA (1990); Bruner et al. (1991a); Booman et al. (1988, 1989); Bagley et al. (in press); Goldberg and Silber (1992)
Mammalian Cell Culture	
Cell Detachment	Bracher et al. (1987); PMA (1990)
Colony Formation	North-Root et al. (1985); Borenfreund and Borrero (1984); Sasaki et al. (1991); Oda et al. (1993)
Chromium Release	Shadduck et al. (1985); Booman et al. (1988, 1989); PMA (1990)
Enzyme Leakage	Grant et al. (1992); Marinovich et al. (1993); Scaife (1985)
Fluorescent Dye Incorporation	PMA (1990); Kemp et al. (1993); Scaife (1985); Bracher et al. (1987); Vaughan and Porter (1993)
Growth Inhibition	Bracher et al. (1987); Kennah et al. (1989a); PMA (1990); Oda et al. (1993)
Highest Tolerated Dose (HTD)	PMA (1990); Borenfreund and Borrero (1984); Shopsis et al. (1985); Riddell et al. (1986); Booman et al. (1988); Goldberg and Silber (1992); Bagley et al. (in press)
MTT Dye	Sina et al. (1992); Nagami and Maki (1993); Reece and Rozen (1993); Grant et al. (1992)
Neutral Red	Goldberg and Silber (1992); Gettings (1993); Bruner et al. (1991a); Riddell et al. (1986); Sina et al. (1992); Bracher et al. (1987); Rougier et al. (1992); Booman et al. (1988, 1989); PMA (1990); Grant et al. (1992)
PGE_2 /ILIa	Cohen et al. (1991); Goldberg and Silber (1992)
Plasminogen Activation	Grant et al. (1992); PMA (1990); Booman et al. (1988, 1989); Bagley et al. (in press)
Protein Inhibition/Denaturation	Bruner et al. (1991a); Marinovich et al. (1993); Rougier et al. (1992); Bernhofer et al. (1993); Sina et al. (1992); Riddell et al. (1986); PMA (1990); Bagley et al. (in press); Booman et al. (1988, 1989)
Uridine Uptake	Shopsis et al. (1985); Booman et al. (1988, 1989); PMA (1990)
Primary Cell Culture	Goldberg and Silber (1992); Shadduck et al. (1985); Grant et al. (1992); Borenfreund and Borrero (1984)
RBC Lysis/Methemoglobin	Muir et al. (1983); Pape et al. (1987); Pape and Hoppe (1990); Sugai et al. (1991b); Lewis et al. (1993); Gettings (1993); PMA (1990)
SAR/Physicochemical	Goldberg and Silber (1992); PMA (1990); Ernst and Arditti (1980); Sugaï et al. (1990); Ptchelintsev et al. (1993)
Silicon Microphysiometer	Cartroux et al. (1993a, 1993b); Rougier et al. (1992); Bruner et al; (1991a, 1991b); Gettings (1993); Goldberg and Silver (1992)
Three Dimensional Models	McCulley and Stephens (1993); Goldberg and Silber (1992); Gettings (1993); Bagley et al. (in press)
Wound Closure	Goldberg and Silber (1993); PMA (1990); Simmons et al. (1987)

The initial search included references cited in Medline and Toxline databases from 1988 to the present. Abstracts were purused, and reprints obtained for pertinent articles. Additional references were obtained from these manuscripts, and also from personal files, to supplement the search references. Only English language references were used in the review.

Results

Seventy-six references were reviewed. A number of excellent overviews of the status of in vitro eye irritation testing procedures have been prepared (see above) by various authors or organizations since Frazier et al. (1987) published their comprehensive "A Critical Evaluation of Alternatives to Acute Ocular Irritation Testing". The contents and emphases in these overviews varied greatly, but the conclusions were amazingly consistent: (1) there is as yet no replacement for the Draize eye irritation test, and the need to find alternative methodology depends on the user's goals and objectives, and the perceived value of the alternative procedure; (2) although great strides have been made in the development of alternative testing procedures, insufficient consideration has often been given to their intended use; and (3) it is generally conceded that the Draize eye irritation test can be replaced in the near future only by a battery of non-animal tests, not by any single procedure.

Nevertheless, the pace of developing non-animal methods for assaying or predicting eye irritation has not slackened; in fact it has increased from 34 (Frazier et al., 1987) to over 60 methods (Atkinson, et al., 1992). The in vitro test systems and references that were reviewed for this manuscript are categorized in Table 1. The most numerous references refer to mammalian cell (mostly monolayer) culture systems. Next in order of frequency are procedures which involve eye components or the entire enucleated eye. Results and conclusions, as well as pertinent comments from cited references and the reviewer, are given below for each of the test systems.

Review of test systems

Agarose diffusion: This assay measures the cytotoxicity of the test sample following its diffusion in an agarose culture medium containing mammalian cells. The diameter of the zone of lysed (non-stained) cells around the sample determines the degree of toxicity. This procedure does not appear to give consistent results that correlate well with the Draize eye irritation test. Rougier et al. (1992) examined 41 surfactants, lotions and shampoos (concentrations not given) in six to eight in vitro procedures; the results indicated that the agarose diffusion assay, using V79 Chinese hamster lung fibroblasts exposed to test substances for 18 hours, gave the worst correlations with in vivo data of any of the eight tests used (the Spearman coefficient comparing the Draize maximum average score and the agarose diffusion procedure for 20 surfactants was < 0.5). The author hypothesized that the diffusion process was the limiting factor and the cause of the poor correlation. Similar poor results were achieved by Bernhofer et al. (1993) when they attempted to distinguish the ocular irritation potentials of 17 mild shampoo prototypes (full strength) by using the agarose diffusion procedure and L929 murine fibroblasts. The authors of

the cited references and the reviewer conclude that the agarose diffusion method is not a viable alternative to the Draize eye irritation test for predicting the corneal responses to products such as soaps and detergents.

CAM / HET-CAM: The chorioallantoic membrane (CAM) or the hen egg test-chorioallantoic membrane (HET-CAM) tests are variations of the same test system: the fertile chicken egg. This system is technically an in vivo procedure because an intact live embryo is involved. However, by using 10-day fertilized eggs, pending European Union restrictions against the use of live embryos is averted. The CAM test is considered to be extremely useful because it is the only "in vitro" test system that can be used to evaluate the irritation potential of virtually any water-soluble or insoluble substance on blood vessels, which play a critical role in the inflammatory process in vivo (Goldberg and Silber, 1992). The test is performed by applying a small quantity of the test substance to the chorioallantoic membrane, which is easily visualized by removal the egg shell and membranes around the air pocket. The injection, hemorrhage and coagulation (necrosis) is then graded over a prescribed period of time, usually 0.5 to 5.0 minutes.

Rougier et al. (1992) found that the HET-CAM test provided the highest correlation between the Draize eye irritation test maximum average scores (MAS) and any of the eight in vitro tests used to evaluate ocular irritation of 41 surfactants and cosmetic products (Spearman correlation = 0.96 for all products; 0.98 for the 20 surfactants). Spielmann et al. (1993) reported similar results from interlaboratory tests in Germany where 32 chemicals from a variety of chemical classes were compared. The authors state that the evaluation of a total of 200 chemicals tested during the validation study indicates a positive predictive value of the HET-CAM test and poor correlation between cytotoxicity assays and in vivo data.

Studies performed under the auspices of the Soap and Detergent Association (Bagley et al., in press) also indicate that the chorioallantoic membrance vascular assay produced good correlations with the Draize eye irritation test (correlation coefficient = -0.91): the correlation also was not affected by alkaline test materials. All 22 test products were correctly classified as irritants or non-irritants according to Federal Hazardous Substances Act (FHSA) criteria.

In contrast to the results cited above, several references indicated that the CAM test system was not of great value. The Pharmaceuticals Manufacturers Association (PMA) Drug Safety Subsection In Vitro Toxicology Task Force (1990), in their evaluation of the various alternatives to the Draize eye irritation test, classified the CAM test as "inadequate". This evaluation was based on the following criteria: (1) the CAM test is not a true in vitro test but an in vivo test; (2) the CAM test, although reported to mimic inflammatory potential, does not possess characteristic features of the inflammatory response observed in the eye, and (3) relatively poor correlations with in vivo results. A similar conclusion was reached by Bruner et al. (1991a) when 17 materials, including chemicals, household cleaners, soaps/detergents and shampoos, were evaluated by in vitro methods and compared to the Draize eye irritation test. The test used by these investigators was the BE/ CAM, which is a combination test using enucleated bovine eyes

as well as CAM. Nevertheless, there was little relationship between the in vivo irritancy potential and the in vitro endpoint for the 17 test materials. Whether the exposure used by these investigators was optimal (200 microliters of test substance for an exposure of only 20 seconds) is not known, but the results prompted the conclusion that the BE/CAM is not useful for assessing the irritant potential of test materials similar to those studied. Furthermore, some researchers have questioned the acceptability of the CAM as a model for eye irritation because it lacks a classic inflammatory response, and is subject to a high rate of chick mortality and false positive reactions.

Based on the literature reviewed, the present reviewer is of the opinion that the CAM model or one of its variations, HET-CAM or CAMVA (chorioallantoic membrane vascular assay), has substantial merit and should be included as part of an in vitro battery to evaluate potential eye irritancy. As stated previously, it is the only system presently useful for mimicking the conjunctival portion of the Draize test by determining irritant effects on blood vessels. Also, the excellent results obtained in interlaboratory studies by the Soap and Detergent Association and in Germany tend to indicate that the CAM test is a useful tool which should be pursued further. An objective, rather than a subjective grading method would be a helpful modification to the procedure.

Enucleated eye tests (EET): This class of assays includes procedures that involve the exposure of enucleated eyes from various species (bovine, chicken, pig, rabbit) as well as the use of eye components such as corneas and lenses. Even the optical sensitivity of bovine lenses, as determined by laser scans, has been tested (Sivak et al., 1992). The PMA Drug Safety Subsection In Vitro Toxicology Task Force (1990) identified these tests as potential alternatives to the current Draize eye irritation test because the assays were considered to be mechanistically valid by virtue of their use of eye tissue and because the measurements were either biochemically relevant to eye physiology or were used in current in vivo procedures. Although there are a number of variations in use, the general procedure is to place the eyeball, or the corneal portion of it, in a suitable holder, place it into an incubator, superfuse it with physiological fluids, expose it to the test agent for a specified time, rinse the exposed surface and grade the injury produced by the agent. Corneal swelling and/or opacity and altered penetration of fluorescein dye through the cornea are the typical parameters that are investigated. Price and Andrews (1985) in fact advocated an in vitro test in which the corneal thickness (swelling) of enucleated rabbit eyes was periodically measured by a photoslit-lamp microscope over a five hour period. Based on tests with 60 widely diversified chemicals, the test was considered to be a reliable alternative to the Draize eye irritation test. However, the assay required six eyeballs per compound tested; therefore, little if any, reduction in animal usage would be possible, which is a major disadvantage.

The relationship of corneal swelling and corneal opacity was investigated by Igarashi and Northover (1987) in bovine isolated corneas exposed to eleven chemicals (sodium decyl sulphate, sodium lauryl sulfate, triethanolamine decyl sulfate, lauryl trimethylammonium bromide, myristyl

trimethylammonium bromide, Tween 20 and 80, ethanol, propylene glycol, dioxane, carbitol and allyl alcohol). A good correlation between the Draize eye irritation test and the bovine EET was achieved. However, the correlation between corneal swelling and opacity was tenuous after treatment with anionic and nonionic surfactants and some of the chemicals, while no correlation existed at all after treatment with cationic surfactants. Because cationic surfactants are more irritating in vivo than anionic surfactants (cause more opacification), it was concluded that the measurement of corneal opacity is a more reliable indication of potential in vivo irritancy than is the measurement of corneal thickness.

The use of rabbit eyes for in vitro studies was probably a logical extension of the Draize eye irritation test and an attempt to minimize animal suffering. With this in mind, Burton et al. (1981) proposed an in vitro assessment of severe eye irritants. The following 11 irritating chemicals were tested in vitro by exposing the corneal surface of eyeballs to two drops or 50 mg of test substance for 10 seconds: 1N sodium hydroxide, 40% formaldehyde, acetic anhydride, allyl alcohol, n-butanol, ethanol, acetone, cetyl trimethyl ammonium bromide, toluene, propylene glycol and glycerine. When compared to published results achieved in the Draize eye irritation test, there was good agreement between the two methods as far as the three most irritating and the two least irritating substances were concerned, but the correlation was poor for solvents.

In more recent in vitro screening efforts the rabbit has been largely replaced by the bovine EET because bovine eyes can be obtained from slaughterhouses, while rabbits must still be sacrificed to obtain their eyes. The techniques have also become more sophisticated with time. A more objective and accurate method of measuring corneal opacity was described by Muir (1984), who used an instrument called an opacitometer. He compared the opacity of the corneas from enucleated eyes exposed to one of two anionic surfactants, sodium lauryl sulfate (NaLS) or sodium decyl sulfate (NaDS), at concentrations of 0.001M. Readings were taken at 4, 8 and 24 hours. The NaDS acted more rapidly and with greater potency that the NaLS, a result that agrees with in vivo findings but not with in vitro cytotoxicity. The same bovine EET system was also used to determine the irritancy of eight anionic surfactants and five industrial chemicals (Muir, 1985). At concentrations of 0.01 to 0.0001M, the short chain surfactants within each analogue series were the most irritating, a finding that corresponded to in vivo results.

The bovine EET was also used as one of six to eight in vitro procedures used by Rougier et al., (1992) to study the ocular safety assessment of 41 surfactants, lotions and shampoos. However, after an exposure of 10 minutes to the test substance (presumably in neat form), the corneas were not only examined via an opacitometer, but also subjected to a fluorescein permeability (penetration) test. The test produced an overall Spearman correlation coefficient of 0.77 when a comparison was made with the Draize MAS. Furthermore, it was concluded that in the case of the irritating surfactants, opacification of the cornea was an important feature that appeared to be relevant to recovery of the damaged tissue. Gautheron et al., (1992) essentially produced the same results when they tested 44 common chemicals via the bovine EET. Their conclusions state that the two endpoints in the bovine corneal opacity and permeability assay are sufficient to accurately predict ocular irritancy. The conclusion was verified with 15 process intermediates.

Nevertheless, better differentiation between mild and moderate irritants was considered desirable. Further verification of the applicability of the bovine corneal opacity-permeability assay was documented by Stankowski et al. (1993) and Vanparys et al. (1993). The latter researchers tested 50 compounds selected on the basis of their previous in vivo irritation potential. When the compounds were classified as irritants or non-irritants, 96% of the chemicals were classified correctly by the bovine EET. When four classes were established (non-irritant, mild, moderate and severe irritant), a 72% concordance was obtained.

The bovine EET can also be used in combination with another assay. For instance, the BE/CAM (bovine eye/chorioallantoic membrane) test is a combination assay that uses freshly isolated bovine eyes to assess corneal damage, and isolated chicken chorioallantoic membranes to assess potential conjunctival irritancy. Bruner et al., (1991) used this combination as one of seven tests to assess the ocular irritancy potential of 17 test materials (mostly soaps and detergent products at various concentrations). The exposure time in the BE assay was 30 seconds, and in the CAM assay, 20 seconds. The extent of the eye injury was determined by visually grading opacity (5 grades), epithelial integrity (4 grades) and epithelial detachment (4 grades). The highest score from either the BE or the CAM was assigned to the substance tested. All but two of the 17 substances tested were classified as slight irritants by this assay, despite the fact that the MAS from the low volume eye test (LVET) ranged from 0 to 48. It was concluded by the authors that the BE/CAM assay, as run, is not a useful tool for assessing the ocular irritancy of substances like the ones tested. The poor correlation is somewhat surprising in view of the rather favorable assessments given to the bovine eye test in other references included in this literature search. Review of the published data did not reveal the cause of the problem.

The most recent EET is the chicken enucleated eye test, the CEET. According to Prinsen (1993), this test has a number of advantages over other in vitro assays: the eyes are readily available in large quantity from slaughterhouses; the chicken cornea is claimed to be more similar to the human cornea than is the rabbit cornea; both humans and chickens have well developed Bowman's membranes; and the test is rapid. Corneas are exposed to test samples of 30 microliters or 30 mg for 10 seconds, after which the corneas are periodically examined for four hours by slit-lamp microscope. Mean scores for corneal swelling, opacity and fluorescein retention determine the irritancy potential. A comparative study with 21 compounds revealed the CEET to be a reliable and accurate predictor of ocular irritancy. When compared to the European-Countries in vivo classification, the CEET correctly classified all 21 compounds (Prinsen and Koeter, 1993).

Whether species-related morphological differences are of any practical significance in the various enucleated eye tests has not yet been determined. To shed some light on this intriguing subject, Berry and Easty (1993) compared the effects of nine irritating chemicals on enucleated human and rabbit eyes. The human eyes, donated for transplantation and research, were not suitable for clinical use. The rabbit eyes were obtained and stored under conditions similar to those used for the human specimens. Corneal thickness (swelling), corneal appearance, condition of the epithelium and fluorescein penetration of the cornea were assessed after exposure to one of the chemicals (in quantities of 20 or 100 microliters for 10 or 60 seconds, respectively). The effects

of allyl alcohol, Arlagard E, 1N sodium hydroxide, sodium chloride, 2% sodium lauryl sulfate and Tween-20 were similar in human and rabbit eyes. Acetone, ethanol and glycerol had opposing effects on corneal thickness in human and rabbit eyes. It was concluded that substances in which penetration depends on corneal surface properties may have species-specific effects. In vitro preparations of human, pig and rabbit corneas were also used by Nuyts et al. (1990) to explore the cause of an accidental toxic endothelial cell destruction syndrome in 18 patients who had undergone intraocular surgery. Concentrations of 1% or greater of a nonionic detergent produced similar endothelial cell damage in both the human and porcine corneas. In another experiment, similar corneal swelling was produced in the human and rabbit corneas after exposure to 1% concentrations of the detergent.

After evaluating all the references pertaining to the EET in this literature search, the reviewer made the following conclusions: (1) a variation of the EET appears to be a prudent inclusion in an in vitro ocular irritation screening battery; (2) eyes appear to behave in rather similar fashion when exposed to irritants, regardless of species, and therefore the morphologic differences (and species differences) would appear to be of secondary importance; (3) differences in technique or methodology appear to play the predominant role in determining whether a particular category of assays such as the EET can achieve consistent results; and finally (4) the chicken enucleated eye test (CEET) appears to be an extremely intriguing assay with multiple advantages, although lack of any peer reviewed publications on this procedure tends to temper excessive enthusiasm but should encourage further comparative studies.

EYTEX (protein matrix assay): This assay has also been called the target biomacromolecular assay because the system utilizes quantifiable alterations in key macromolecules as toxicological endpoints. An excellent short history and overview of this procedure has been published (Rowan and Andrutis, 1989). This report states that several series of validation experiments, using a broad range of household chemicals, soaps, detergents and cosmetics, have produced overall correlations of >89% when compared with published results achieved with the Draize eye irritation test (no references given). The theory and mechanistics of the test are well summarized by Gordon (1992), one of the developers of EYTEX. According to this author, the method is based on alterations in the conformation and hydration of an ordered macromolecular matrix, the major part of which is a high molecular mass oligomeric protein. The delivery system and reagent have been combined with an instrument system for standardization. Changes in optical density of the matrix reflect the degree of "toxicity" produced by exposure to the test substance. Four protocols are currently available so that materials of different physicochemical properties and sensitivities can be evaluated. These new protocols have replaced the earlier Standard Assay and Membrane Partition Assay.

Gordon (1992) reported the results of 100 diverse chemicals studied by the EYTEX method. The chemicals ranged from nonirritating to extremely irritating and included solutions, insolubles, solids and colored materials. Samples were analyzed at three dose levels; surfactants were studied at 1% and 10%. The test substances were classified into five categories (minimal, mild, moderate,

severe or extreme irritants) and an EYTEX/Draize Equivalent (EDE) was calculated. An equivalence of 91% between in vivo and in vitro results was calculated; however, no correlation coefficients were given. The author concluded that the sensitivity (ability to correctly identify irritants) of 98% that was achieved in this study indicated that the EYTEX method has utility as a primary ocular screen of chemicals.

The EYTEX in vitro assay was used by Decker and Harper (1993) to evaluate 42 adult and baby shampoos. Ethoxylated and nonethoxylated alkyl sulfate surfactants were the primary ingredients of the adult shampoos, while branched-chain ethoxylated alkyl, as well as amphoteric surfactants were the main components of the baby shampoos. All shampoos were diluted 1:10 with deionized water and tested in two or three separate experiments. The correlation of the Draize scores to the EYTEX scores was statistically significant ($r^2 = 0.90$). When the scores were used to classify the test agents into minimal, mild, moderate and severe irritants, the Draize and EYTEX classifications correlated 100% for the baby shampoos and 87% for the adult shampoos. The authors concluded that for the shampoos studied, the EYTEX system of testing for ocular irritancy is highly predictive of the maximum 24-hour Draize eye irritation scores.

The EYTEX system was also used in the following studies in which various in vitro test procedures were evaluated. According to a recent review by McCulley and Stephens (1993), 465 cosmetic product formulations and raw ingredients were evaluated with EYTEX, and the results were published in ATLA in 1992. The ability of EYTEX to detect ocular irritants varied with the type of test and product class. In addition, Gettings (1993) has indicated that a three-phase assessment of 25 in vitro alternatives to the Draize test, sponsored by the Cosmetic, Toiletry and Fragrance Association (CTFA), is nearing completion. The EYTEX system was one of six tests that was shown to have the most agreement with the Draize eye irritation test.

However, not all researchers agree on the usefulness of the EYTEX system. The PMA Drug Safety Subsection In Vitro Toxicology Task Force (1990) indicated in their review that protein matrix assays were "inadequate", and that the physicochemical reaction was irrelevant to the eye. There were evidently also some reservations by researchers in the soap and detergent industry because the EYTEX assay was not included in the extensive SDA three-phase in vitro methods assessment program. Furthermore, a review of the references from the present literature search reveals that not all of the users of the EYTEX system obtained consistent results. Bruner et al. (1991a), in their evaluation of 17 surfactant-based chemicals and consumer products by seven in vitro alternatives for ocular safety testing, found that there was no correlation between the in vivo LVET MAS and any of the endpoints obtained by the EYTEX system ($r = -0.29$). The completely random relationship between the in vivo and the EYTEX results could not be explained by the mere fact that low volume eye test results were used for comparison purposes rather than the regular volume, maximum 24-hour Draize eye irritation test results. In yet another study, in which six to eight in vitro assays were used to test 41 surfactants, lotions and shampoos (Rougier et al., 1992), the EYTEX assay had the lowest correlation coefficient ($r = 0.74$) of any of the six tests for which comparisons were calculated to the Draize MAS data. As a result, the authors did not recommend the EYTEX system for ocular screening.

Obviously conflicting EYTEX results were obtained by researchers cited in this literature review. The degree of the inconsistency was remarkable, but the reason for it could not be determined. Variations in technique certainly cannot be ruled out, and careful interpretation of the statistical methods used to determine the necessary Draize eye test correlations may resolve some of the inconsistencies. But, as pointed out in several reviews (McCulley and Stephens, 1993; PMA, 1990), multiple mechanisms are involved in corneal and lenticular damage induced by chemical substances with widely diverse properties; therefore, a test based only on the denaturation of protein may not always be pertinent.

Inverebrate models: Two invertebrate models for the in vitro evaluation of ocular irritants were revealed in the present literature search. One system, the luminescent bacteria toxicity test (LBT), assesses changes in light output from the luminescent bacteria *Photobacterium phosphoreum* as a nonspecific indicator of toxicity. The system is available in commercial kits under the name of MICROTOX. When Bruner et al. (1991a) investigated 17 chemicals and surfactant-based preparations for potential ocular irritancy by seven in vitro procedures, the LBT was performed on serial dilutions of the test substances, which were then exposed to the bacteria for five minutes. Light output from the bacteria was measured photometrically before and after exposure, and the concentration of test material that decreased the light output by 50% was used as the end point. The LBT data ranged over approximately four orders of magnitude and the correlation coefficient between the in vitro and the LVET MAS was significant ($r = -0.91$).

The LBT was also used as one of six to eight in vitro tests to evaluate 41 surfactants, lotions and shampoos (Rougier et al., 1992). The results obtained were correlated with Draize MAS and found to be significant ($rs = 0.79$). Although the data obtained by this test were considered encouraging, the LBT was not selected for further testing by these researchers because the biological system was not as relevant to in vivo ocular irritation as other cell culture techniques that gave better or equivalent correlations. In another study, the MICROTOX system was used to evaluate a series of 20 cosmetic surfactant-containing products and their constituent surfactants and preservatives for potential ocular irritancy (Miller, et al.,1993). The authors summarized their findings by stating that the MICROTOX system in general was able to predict correctly the irritation potential of individual surfactants and the very mild and the very irritating surfactant-containing products. However, the irritation potential of the moderate irritants and the preservatives was not well predicted.

The second invertebrate system examined in this review was the tetrahymena/motility assay (TMA). This assay is based on the principle that irritating substances tend to decrease the motility of the ciliated protozoan *tetrahymena thermophila* when the irritants are added the culture suspension. The endpoint of the assay is the concentration of test material that minimally reduces motility upon microscopic examination after a two-minute exposure.

Although the PMA Drug Safety Subsection In Vitro Toxicology Task Force (1990) classified the TMA "inadequate" because there is no scientific rationale relating protozoan lethality to

mammalian toxicity or to eye irritation, a number of studies have revealed that there is a statistically significant correlation between the TMA and the in vivo Draize eye irritation test results. In the study of 17 chemicals and surfactant-based products (Bruner et al., 1991), a significant correlation between the TMA and the Draize test was obtained (r = 0.78). Significant correlations were also obtained in the three-phase Soap and Detergent Association evaluation. Booman et al. (1988) reported a significance of $p < 0.1$ with eight products in Phase I; In Phase II, Booman et al. (1989) reported only one false positive result after testing 23 products. In the Phase III study, Bagley et al. (in press) reported that the TMA had an overall correlation coefficient of 0.85, and some ability to discriminate Draize test irritants. Only the nonirritant glycerol produced a false positive effect.

In summary, both of the invertebrate tests identified in this literature search may be useful as an in vitro screen for ocular irritation. This conclusion is corroborated by McCulley and Stephens (1993), who stated in their review that the MICROTOX assay was capable of separating irritants from nonirritants, even though the test could not always discriminate the degree of irritation, and by Brunner et al. (1991), who selected both of the invertebrate tests as useful in ocular safety assessment.

Mammalian cell culture models: This class of in vitro procedures contains more references than any of the other classes identified in the present literature search. This should not be surprising because the number of procedural variations that are possible is almost unlimited: the cell lines are of various origins; culture techniques vary; exposure periods vary from reference to reference, as do the concentrations of test substances; and finally the end points differ markedly.

However, before fragmenting this group of references into subdivisions, it may be helpful to the reader to be aware of some of the general comments and conclusions made about the use of in vitro mammalian assays in a few of the more comprehensive reviews from this literature search. For example, the PMA Drug Safety Subsection In Vitro Toxicology Task Force (1990) listed 19 separate mammalian cell culture assays in their review and concluded that they generally represent cellular systems that only measure cytotoxic responses (a common but minor component of eye damage); therefore, they were "tests of questionable relevance". Nevertheless, despite their presumed inability to predict the entire process of eye irritation, and thus replace the Draize assay, such tests were considered by PMA to have some utility in establishing a prescreen hierarchy.

McCulley and Stephens (1993) in their review point out that monolayer tissue culture procedures provide a cost effective approach to assessing ocular irritation; however, there are certain disadvantages to their use. These include the difficulty of testing powders and anhydrous materials, and the existence of a disproportionate relationship between the concentration of a test material required to produce toxicity in vitro and the non-reactiveness of the same concentration in vivo.

Borenfreund and Borrero (1984) also made some interesting observations in their attempts to

evaluate various cytotoxicity assays as potential Draize alternatives. They observed that in their study of four different cell lines (Balb/c 3T3 murine fibroblasts, Chinese hamster lung cells, human hepatoma cells and mouse macrophage cultures) that a good correlation in cytotoxicity rankings existed between cell lines, regardless of the type of cell used. Furthermore, the 50% inhibition of colony formation and the highest tolerated dose (HTD) produced the same ranking of the various test materials. It was concluded by the two researchers that basic cytotoxicity was a useful endpoint in ocular toxicity assays using established cell lines as targets, and that regardless of the cell type examined in the HTD cytotoxicity assay, the ranking of 22 chemicals and 12 surfactants was practically the same.

An alphabetical listing of reviewed cell culture endpoints is given below.

Cell detachment: This assay entails a microscopic evaluation of the number of cells that are detached from the monolayer culture. The endpoint is usually some percentage of the total cells counted. Using an endpoint of 25% cell detachment, a large number of false predictions (especially false negatives) were registered in a study of 26 different cosmetic ingredients (Bracher et al., 1987). The BHK 21/C13 cell cultures were exposed to various concentrations of test substance for four hours; the CD-25 rankings then were compared to an in vivo Draize ranking performed in guinea pigs. Based on the results from this reference, cell detachment does not appear to be a sensitive or consistent cytotoxicity endpoint for measuring potential in vivo eye irritancy.

Colony formation: Cytotoxicity is quantitated by determination of colony-forming ability after exposure to test material (usually one hour). The concentration of the test material required to reduce colony formation by 50% (LC50) is the usual endpoint that is compared with in vivo data. Six shampoos were tested by this method in cultures of SIRC rabbit corneal cells (1% concentration or less of test substance with an exposure time of one hour); a perfect ranking agreement was achieved when compared to results obtained by the Draize eye irritation test (North-Root et al., 1985). The authors concluded that the SIRC cell toxicity test produced a significant correlation with rabbit eye irritation in vivo and that it was a suitable model system for screening shampoos.

The colony formation endpoint was also used to rank four alcohols (butanol, pentanol, ethanol and isopropanol), as well as 34 test agents, including alcohols, ethers, esters, ketones, acids, amides and detergents (Borenfreund and Borrero, 1984). The ID50 (50% inhibition) of colony formation was found to be the same as that determined by the HTD. Because the colony formation test is more laborious, it was concluded that this test could be used as a supplementary confirmatory test to the HTD plate assay.

Fifty-two chemicals (including detergents, glycols, oils and antiseptics) were compared using a colony-formation assay in three stablished cell lines (Balb 3T3 mouse whole embryo, ARLJ301-3 rat liver and FRSK rat keratinocytes); the toxicity ranking was the same as that obtained from Draize eye irritation test data, the correlation coefficients ranged from about

0.6- 0.7 for the four cell types (Sasaki et al., 1991). The exposure time was 6-7 days; the exact concentrations of test materials were not given. However, the degree of cytotoxicity (cationic detergents > anionic detergents > nonionic detergents > glycols or oil) corresponded to results obtained by other researchers.

In another type of assay, irreversible changes in the growth and reproduction of Chinese hamster ovary cells (CHO clonal assay) was used as an endpoint to evaluate the cytotoxicity of benzalkonium chloride and various homologs (Oda et al., 1993). Increasing or decreasing the length of the alkyl chain from 16 decreased cytotoxicity.

The references reviewed appear to indicate that acceptable correlations between the Draize eye irritation scores and the colony-formation inhibition endpoint could be achieved for ranking chemicals. In addition, the cell type used did not appear to be crucial, and similar results were observed regardless of cell line. However, the colony formation assay is about seven days longer in duration and gives essentially comparable cytotoxicity information as other less laborious procedures. Therefore, this assay does not appear to offer any advantages over other methods and cannot be recommended as one of the choice assays for an in vitro eye irritation battery.

Chromium release: In this procedure, cells are loaded with ^{51}Cr and then exposed to test substances. The leakage of ^{51}Cr as monitored with a gamma counter, is a measure of cell death. Good agreement in rankings were achieved between in vivo LVET results and the chromium release endpoint when two murine cell lines in suspension (YAC-1and P815) and a rabbit adherent cell line (SIRC), as well as a primary rabbit corneal epithelial cell culture, were used to evaluate benzalkonium chloride, sodium dodecyl sulfate, two alkylethoxylated sulfates, polysorbate 20 and coconut soap for potential eye irritation (Shadduck et al., 1985). Polysorbate 20 was the only substance which could not be ranked correctly (in vitro results overestimated the toxicity). Otherwise, results were consistent and reproducible. Suspension cultures of murine origin were concluded to be suitable for cytotoxicity assays. The rabbit cells, being adherent, were harder to label uniformly and therefore produced results that were somewhat more variable than the murine data.

When the chromium release system was used in the Phase I Soap and Detergent Association evaluation of eight products, correlation with the Draize MAS data was achieved but non-irritating samples were not always correctly identified (Booman et al., 1988). Using the chromium release endpoint with cultures of P815 murine mastocytoma cells, 8/8 FHSA irritants and 13/15 nonirritants were correctly identified in Phase II of the SDA evaluation of 23 products (Booman et al., 1989).

Based on the above cited studies, it is concluded that the chromium release endpoint is an acceptable method of evaluating cell death in mammalian cell culture systems. However, this method has no particular advantage over other simpler procedures and has the disadvantage of using radiolabeled material. In addition, data from the Phase II SDA study (Booman et al.,

1989) indicate that the reproducibility of the method is lower than in 4/6 other assays (it was not used in Phases I and III). Therefore, this endpoint does not appear to be one that can be recommended as an assay of choice for an in vitro eye irritation battery.

Enzyme leakage/ LDH determination: Measurement of various cellular enzymes has been used by a number of researchers to determine mammalian cell cytotoxicity in vitro. Increased media levels of enzymes such as lactate dehydrogenase and alkaline phosphatase can be used as an indication of increased cell membrane permeability and/or altered cell metabolism.

Eight surfactants were compared and ranked correctly according to their in vivo cytotoxic potential (cationic > anionic = amphoteric > nonionic) when the in vitro cytotoxicity was determined in primary cultures of rabbit corneal epithelial cells by measuring LDH leakage into the culture media after a one-hour exposure period to three concentrations of test agent (Grant et al., 1992). In addition, elevated levels of LDH leakage 24 hours after insult correctly identified and differentiated the strong irritants from the mild ones.

A rank correlation (r = 0.657) was also observed between in vivo and in vitro (murine epidermal cell line HEL 30) toxicity when LDH leakage was used as an endpoint to rank a number of shampoos and their ingredients (Marinovich et al., 1993). The cells were exposed to concentrations of 0.05 to 0.5% (shampoos) and 1 to 10,000 micrograms/ml (surfactants) for an exposure time of two hours. The names and numbers of shampoos were not given.

The in vitro cytotoxicity of a homologous series of purified detergents was assessed in HeLa cells by a test for alkaline phosphatase release (Scaife, 1985). Although in general the decreasing order of potency of cationic, anionic and nonionic detergents was similar in vivo and in vitro, complete agreement was not achieved using cytotoxicity as an endpoint. It was hypothesized that the inconsistencies were due to differing penetration characteristics of the detergents. One of the author's conclusions was that a simple measure of detergent cytotoxicity is not an adequate predictor of ocular toxicity.

Review of the limited number of references on enzyme leakage as a viable endpoint for cytotoxicity leads to the conclusion that this parameter is not currently one of the better in vitro indexes of cytotoxicity. LDH measurement would appear to have some benefit because it may be possible to determine which agents produce acute or prolonged membrane damage, but such information is probably only of supplementary value.

Fluorescent dye incorporation (FDA/EB): This endpoint utilizes the dyes fluorescein diacetate and ethidium bromide to indicate cell viability by assessment of cell membrane integrity and intracellular esterase activity. The cells are exposed to the test substance and dye for a short time and then examined microscopically or by flow cytometry. Intact cells take up the fluorescein diacetate and hydrolize it to fluorescein (green fluorescence), while impaired cells take up only the ethidium bromide, which stains the nuclear DNA red.

The above method was used by Kemp et al. (1993) to determine the ocular irritancy potential of eleven detergent-based products (products not identified) in LS cells derived from NCTC L929 mouse fibroblasts. The cells were exposed for four hours to appropriately diluted (exact dilutions not given) test agent and then counted under a fluorescence microscope. The compounds were ranked by the calculated concentration that produced 50% cell death (CD_{50}). The rankings were then compared to results from the Draize eye irritation test. All samples with a CD_{50}< 0.5 mg/ 0.1 ml were severe eye irritants while, with one exception, all compounds with CD_{50} > 1.0 mg/ 0.1 ml were mild irritants according to the Draize eye irritation test. Cultured cells in suspension were used because established cell lines produce uniform results and suspended cells require less exposure time and are more easily counted. The test was concluded to be a good screening procedure but not an alternative for the Draize eye irritation test.

Scaife (1985), in his evaluation of the cytotoxicity (as determined in suspension cultures of mouse fibroblasts by FDA/EB after four hours of exposure to test substance) of a series of completely soluble detergent-based formulations, also found the FDA/EB method acceptable for screening. However, based on the evaluation of a homologous series of purified detergents exposed to monolayer cultures of HEp2 cells for one hour, the author warned that significant non-correlations may result if the physicochemical characteristics of the test substances differ in the in vitro and the in vivo investigations.

When 26 cosmetic ingredients were evaluated in freshly isolated rat thymocytes, the concentration of test compound that resulted in 25% viability, as determined by the FDA/EB endpoint via flow-cytometry, produced both false negative and false positive reactions when the data were compared to results obtained by the Draize scoring method in guinea pigs (Bracher et al., 1987). Only 12/26 preparations could be tested because of solubility problems, and it was concluded by these researchers that the FDA/EB test was not as predictive nor as sensitive as the neutral red test.

Acridine orange (AO) and propidium iodide (PI) can also be used to determine cell viability. When examined microscopically under fluorescent illumination, the AO stains viable cells green and the PI stains dead cells red. Vaughan and Porter (1993) used suspensions of L-929 mouse fibroblasts and this methodology to assess the cytotoxicity of various contact lens-care solutions. Benzalkonium chloride (0.1% and 0.01%) was found to be highly cytotoxic, as were solutions of two ionic surfactants (0.1% sodium dodecyl sulfate and 1% sodium tridecyl sulfate) and two nonionic surfactants (1% Tween-20 and P-40). Unneutralized hydrogen peroxide (3%) and some of the other disinfectants/preservatives also produced various degrees of toxicity. No comparisons were made between the in vitro and the Draize eye irritation test.

The conclusion drawn from the above citations is that fluorescent dyes can be used successfully to determine the degree of cell viability in culture. Suspension cultures are claimed to produce better results with this method than monolayer cultures. The type of cell used does not appear to be critical. The dye method does not appear to have any advantage

over other methods such as the neutral red assay.

Growth inhibition: This endpoint can actually be viewed as a variant of cell detachment or colony forming ability. Cells are usually grown under confluent culture conditions, exposed to test substances for 48 hours, and then the number of cells present before and after exposure is compared.

A 50% growth inhibition calculated after exposing BHK21/C13 cells for 48 hours to 26 different cosmetic ingredients produced a large number of false predictions when the results were compared to the Draize-type evaluation in guinea pigs (Bracher et al., 1987). The method was particularly unreliable for the minimally irritating and nonirritating products. The neutral red assay was considered superior to growth inhibition by these researchers.

Kennah et al. (1989a) evaluated 24 chemicals, including six surfactants, seven alcohols, four ketones, four acetates and three aromatics, in an in vitro system using BALB/c 3T3 cells exposed for 30 minutes to the test agents. After 24 hours of incubation the cells were counted and 50% growth inhibition calculated. A significant linear correlation between cytotoxicity and ocular irritancy was established only for surfactants and alcohols; for the other substances there was little correlation. The author concluded that in vitro cytotoxicity data alone cannot be used to predict ocular irritancy of a broad spectrum of chemicals.

Based on the two references cited above, growth inhibition *per se* does not appear to be a particularly attractive endpoint for the in vitro evaluation of cytotoxicity. Better correlations with in vivo evaluations are achieved with other techniques.

Highest tolerated dose (HTD/IC50): This endpoint relies on the morphological assessment of cells in semiconfluent cultures after exposure (usually 24 hours) to test substances. The concentration producing minimal morphological change is considered to be the HTD. The observations are subjective and depend upon the experience of the operator.

In a study involving 34 test agents (alcohols, ethers, esters, ketones, acids, amides and detergents), the HTD in five different cell types (epithelial rabbit cornea cells, murine fibroblasts, Chinese hamster lung cells, human hepatoma cells and mouse macrophage cultures) was found to produce virtually the same toxicity ranking for all the agents (Borenfreund and Borrero, 1984). All agents were soluble and the best exposure period was found to be 24 hours. The in vitro rankings compared well with the Draize eye irritation test reported by previous investigators.

The HTD was also determined for 19 diverse chemicals exposed to Balb/c 3T3 cells for 24 hours (Shopsis et al., 1985). Results obtained with surfactants indicated that these substances were more toxic in vitro than in vivo, a result also observed by previous investigators; HTD rankings correlated well with the uridine uptake assay and with in vivo ocular irritancy. Riddell et al. (1986) also obtained consistent results with the HTD endpoint when 30 coded

chemicals were tested in 3T3-L1 cells. The method correlated well with the neutral red uptake and measurement of total cell protein.

The HTD was also used as one of the endpoints in Phase I of the SDA evaluation study (Booman et al., 1988), in which eight detergent-based materials were assessed. The HTD did not correctly classify all the nonirritants, and significant correlations were not obtained with Draize MAS. An HTD assay was not used in Phases II and III of this evaluation, although a fibroblast cytotoxicity EC_{50} assay was conducted (Bagley et al., in press). The EC_{50} was not defined, but the assay produced a correlation coefficient of -0.61 when all of the test materials were considered, which ranked nineth in the battery of eleven assays.

A review of the information cited in the available references indicates that the HTD may vary substantially, depending on the expertise of the investigator, and the lack of an objective end point appears to be a major disadvantage. There are no apparent advantages to compensate for this disadvantage, especially since other endpoints appear to be at least equally effective.

MTT assay: The tetrazolium salt MTT is a water soluble dye that is reduced in active mitochondria to an insoluble blue crystal. The extent of the blue color in a culture is directly proportional to the number of viable cells when measured photometrically after an appropriate exposure to test substances. IC_{50} comparisons can then be calculated.

Using the above technique, Sina et al. (1992) evaluated 27 commercially available compounds and 56 pharmaceutical raw materials or manufacturing intermediates for potential ocular irritation in V79 Chinese hamster cells and also in rabbit corneal epithelial cells. Cell cultures were exposed to the test compounds for a period of 23 hours. The same compounds were also evaluated by the incorporation of leucine into protein and by the neutral red assay. Analysis of the data indicated that none of the assays accurately predicted in vivo ocular irritation, and although the MTT assay gave the best correlation, all Spearman correlation coefficients were < -0.5. It was concluded that the measurement of cytotoxicity is of limited value when testing substances such as the ones used in this study.

Nineteen chemicals, including mostly surfactants and alcohols, were tested for potential ocular irritation in vitro, using normal human epidermal keratinocytes (NHEK) and the MTT assay as an endpoint from which an IC_{50} could be calculated (Nagami and Maki, 1993). The IC_{50} was then compared to the in vivo DS_0, the maximum concentration of test substance which produced no ocular irritation in rabbits according to the Draize eye irritation procedure. A correlation coefficient of 0.82 was established when all 19 substances were considered. It was concluded that this assay could reliably predict in vivo doses that would not produce ocular irritation.

The MTT model was also used in a fibroblast three-dimensional skin model to assess the ocular irritancy of 15 surfactants (Reece and Rozen, 1993). A sensitivity of 83% and a specificity of 88% was established. The exposure period was one hour. Another set of eight

surfactants was evaluated in primary cultures of rabbit corneal epithelial cells, and the MTT assay served as endpoint (Grant et al., 1992). The MTT, as well as the NR, assay was considered to be a good predictor of surfactant cytotoxicity in CE cells. The assays correctly ranked the cytotoxic potential of the surfactants. The prolonged eye damage that occured with severe irritants could not be evaluated by the MTT method.

Based on the data reviewed, the MTT assay is an appropriate method of determining cytotoxicity. The assay does not appear to have any major advantages or disadvantages and test results parallel those of other acceptable methods. The predominant reason for divergent results with this test appears to be the types of compounds tested. As stated repeatedly by various investigators, cytotoxicity may not be a relevant endpoint for all test substances.

Neutral red assay: This assay measures the ability of a test chemical to inhibit the uptake of neutral red dye. Healthy cells accumulate the dye in the lysosomal compartment and the accumulation can be measured spectrophotometrically. As described by Goldberg and Silber (1992), there are two variations of the method available: (1) dye is added to the culture near the end of test substance exposure, which is usually 48 hours, and the amount of NR uptake by the normal human keratinocytes is measured (method is now also available commercially from the Clonetics Corporation); and (2) the cells, normal human epidermal fibroblasts are preloaded with NR and the test substance is added for only 1-5 minutes, after which the retention of dye is measured. The NR assay has been conducted on established cell lines as well as on primary cell cultures.

NR citations in this literature search outnumber any other endpoint used for cytotoxicity evaluation; in many instances the NR assay is the "standard" test used in a battery or the test to which newer or more complex tests are compared. Thus, the NR uptake, as well as the NR release, assays were performed in the CTFA evaluation of Draize eye irritation test alternatives (Gettings,1993); the latter test was shown to be one of six tests that had the best correlation with the Draize eye irritation test. Bruner et al. (1991) also performed both variations in their evaluation of 17 surfactant-based substances by seven procedures. The study indicated that the two NR assays were comparable to each other and produced virtually the same correlation coefficients with the LVET MAS Draize test ($r = -0.85$ and -0.86).

Two references cited the use of NR assays as one of three procedures to evaluate pharmaceutical-related products/ingredients. In one instance, Sina et al. (1992) tested 83 pharmaceutical products and materials and found that there was good agreement among the three in vitro assays (neutral red, MTT and leucine incorporation) used to determine the general cytotoxicity rankings of the compounds. However, it was concluded that neither NR uptake, MTT dye reduction nor measurement of protein, when used in conjunction with primary cell cultures of rabbit corneal epithelial cells, provided adequate predictiveness of ocular irritation. The correlation index between these methods and in vivo eye irritation was < -0.5. In a similar fashion, Riddell et al. (1986) determined that results obtained in a blind trial with 30 chemicals indicated a close correlation between the relative cytotoxicities of

chemicals tested in 3T3 mouse fibroblasts by measurement of NR uptake, total cell protein and morphological evaluation. Unfortunately, correlations between in vitro and in vivo findings were not conducted or even addressed.

When various surfactants were evaluated by the NR assay, close agreement was achieved by three investigators. Grant et al. (1992) reported that the NR, MTT dye reduction and LDH leakage assays all produced similar rankings of surfactants. All were able to evaluate the irritation potential of these materials. Nevertheless, the authors stressed the importance of using several cytotoxicity tests with different endpoints to obtain an integrated perspective of the cytotoxic potential. Bracher et al. (1987) concluded that of the five assays evaluated, only NR uptake could distinguish the minimally irritating test agents from strong, and nonirritants. All three extremely irritating quaternary ammonia compounds were identified as the strongest cytotoxic agents; 9/12 minimally irritating substances were correctly ranked as intermediate irritants, and 8/11 nonirritants were cytotoxic only at extremely high concentrations. Similary in their study of 41 surfactants, lotions and shampoos, Rougier et al. (1992) confirmed the finding that the NR assay was a good endpoint for the evaluation of products with low irritancy potential. In addition, a correlation coefficient of 0.80 was achieved between the NR assay and the results from the Draize MAS.

The neutral red uptake assay also served as an endpoint in the SDA evaluation of alternatives for the Draize eye irritation test; however, the assay did not distinguish itself in either Phase I or Phase II (Booman et al., 1988; Booman et al., 1989). In Phase I, nonirritating systems were correctly identified by only one of the two variants of the NR uptake; no significant correlation with the Draize MAS was achieved. In Phase II, twenty products were evaluated with the NR uptake assay; 12/13 FHSA irritants and 6/7 nonirritants were correctly identified. In Phase III, two variations of the NR uptake assay were again employed in the testing of 22 soap and detergent products. The methods achieved remarkably similar results: correlation coefficients of -0.74 and -0.78 were achieved when all products were considered, and coefficients of -0.88 and -0.92 resulted when only non-alkaline materials were included in the calculation (Bagley et al., in press). However, neither test was able to distinguish among the irritants based on Draize eye irritation scores.

In summary, the neutral red assays appear to have some promise for ranking potential ocular irritants, especially the weak ones. The method is easily standardized; yet, there is quite a lot of "interlaboratory" variation among investigators as to the outcome of their screening trials. A prime factor is the type of product tested; highly soluble products of fairly simple structure appear to be better candidates than more complex pharmaceutical formulations that may be less soluble or even hydrophobic. Nevertheless, this assay should, according to most of the authors, be included in any screening battery for ocular irritants.

<u>PGE2/IL-1a</u> <u>assays</u>: Irritation is related to a set of reactions involving the inflammatory process, and it has been suggested that the in vitro determination of inflammatory parameters might be predictive for the evaluation of ocular irritation. This suggestion is based on the fact

that interleukin 1 (IL_1) and prostaglandin E_2 (PGE_2) are produced by human keratinocytes after UVB and UVA irradiation. The finding was confirmed in vitro by Cohen et al. (1991), who observed intra- and extracellular increases in IL_{1a} and PGE_2 after cultured keratinocytes were exposed to UVB irradiation or sodium dodecyl sulfate. The considerable intertrial variation did not prevent the authors from suggesting that measurement of these mediators may provide an in vitro test to detect potentially irritating products. However, at the present time this assay is not ready for use as a practical screening procedure, and further research is needed to develop this potential alternative procedure.

Plasminogen activation: This assay uses the release of plasminogen activator from primary rabbit corneal epithelial cells as a quantitative index of toxicity. According to Grant et al. (1992), plasminogen activator (PA) is a serine protease that converts plasminogen to the fibrinolytic enzyme plasmin and plays a role in initiating stratification, cytomobility and cellular response to corneal injury. It has a major theoretical advantage in that it uses rabbit corneal epithelial cells, which should provide good correlation with in vivo evaluation. However, according to the PMA review, the actual release of PA is erratic, and it is concluded that although the model may have some merit, the test itself is questionable in terms of how it could be utilized as a predictor of ocular toxicity (PMA Drug Safety Subsection In Vitro Toxicology Task Force, 1990).

Nevertheless, the test has been incorporated into the SDA evaluation of Draize eye irritation test alternatives and in Phase I, the assay correctly classified all nonirritating test agents and correlated significantly with the Draize MAS (Booman et al., 1988). Furthermore, in Phase II of the SDA program the assay correctly classified 10/10 FHSA irritants and 5/5 nonirritants (Booman et al., 1989). The excellent correlation with the Draize scores continued in the Phase III program where the correlation coefficient with all of the test materials was -0.85; the correlation coefficient was not affected by the alkalinity of the test materials (Bagley et al., in press).

At the present time there are not enough data from the references to make any conclusions about this test. However, if the SDA evaluation is an indication of the assay's potential, further work with this procedure is surely warranted.

Protein inhibition/denaturation: Although a number of analyses are used to determine total protein content in the in vitro studies, the methodology used does not appear to be critical. In many of the studies the total protein (TP) is determined right after the neutral red assay has been performed. The rationale for this assay is that interference with any number of factors at the cell level, such as transcription and amino acid uptake or transport, could theoretically interfere with the necessary synthesis of protein. In general, cells are incubated with test material, the cells are then washed and total protein of the incubate is determined. The concentration of chemical required to reduce protein by 50% is the usual endpoint for comparative purposes.

In the evaluation of seven in vitro alternatives for ocular safey testing, Bruner et al. (1991a) compared 17 surfactant-based products and attempted to find the assays that would be most useful. It was concluded that one of the five most useful tests was the determination of total protein because a correlation coefficient of -0.86 was obtained when this assay and the in vivo Draize evaluation were compared. However, the neutral red assay produced parallel results, leading the investigators to conclude that the assays were duplicative and that the NR test was preferred for ocular safety screening in vitro.

Shampoos and/or surfactant preparations were also studied by three additional investigators. Marinovich et al. (1993) tested shampoos and their ingredients in a murine epidermal cell line (HEL 30) using TP and lactate dehydrogenase (LDH) leakage as end points for cytotoxicity. Cells were exposed for two hours to concentrations of 0.05 to 0.5% (shampoos) or 1 to 1000 micrograms/ml (surfactants). The substances were ranked in order of toxicity and results were compared to in vivo Draize scores. Correlation coefficients of 0.657 and 0.524 were obtained for the LDH and TP inhibition assays, respectively. The relatively low coefficients were increased dramatically when anionic surfactants were excluded from the calculations. Similar findings were published by Rougier et al. (1992) in a study of 41 surfactants, lotions and shampoos. V79 Chinese hamster lung fibroblasts were exposed to test substances for 24 hours. Protein inhibition was measured and compared to MAS Draize scores. A correlation coefficient of 0.56 was obtained for surfactants. Protein content was rejected as a test parameter for evaluation of all 41 products. Poor correlations between the TP assay and Draize results was also described in a comparison by Bernhofer et al. (1993) when they attempted to compare results from 17 mild shampoo prototypes (diluted concentrations exposed to cells for two hours) evaluated in primary normal human epidermal keratinocytes. It was concluded that cytotoxicity methods were not capable of predicting the corneal responses to repeated applications of mild shampoo formulations.

The TP assay was also used to predict ocular irritation for pharmaceutical-type products. Sina et al. (1992) compared the MTT, NR and TP assays, using primary cultures of rabbit corneal epithelial cells and V79 Chinese hamster lung fibroblasts exposed for 23 hours to 27 commercially available compounds and 56 in-house intermediary products. TP was determined by leucine incorporation. IC_{50} values were similar between the MTT, NR and the TP values, but when the results were compared to in vivo findings, the correlation coefficient was < -0.5 in all three cases. It was concluded that the cytotoxicity measurements were adequate for ranking compounds within a chemical class but not among classes. The parallelism between results obtained by the TP, NR assays and morphological evaluation was also noted by Riddell et al. (1986) in their study of 30 coded pharmaceutical-type compounds in 3T3-LI mouse cells. No comparisons were made to in vivo studies.

Total protein synthesis (KBR method) was also one of the many parameters examined by the SDA evaluation program for alternatives to the Draize eye irritation test. In Phase I, the TP assay did not identify correctly the nonirritating agents tested, and there was no significant correlation between the results from this assay and the MAS Draize scores (Booman et al.,

1988). In Phase II, the TP assay, performed in normal human epidermal keratinocytes, correctly identified 12/13 FHSA irritants and 6/7 nonirritants (Booman et al., 1989). In the Phase III portion of the program, 22 soap/detergent type products were tested in the various assays, including the TP assay. Correlation with Draize MAS depended upon the alkalinity of the test material. An overall correlation coefficient of -0.74 was calculated when all materials were considered; this improved to -0.88 when alkaline test materials were excluded (Bagley et al., in press). Nevertheless, the assay was not entirely satisfactory because it did not discriminate well between highly irritating substances (three of the most irritating materials in vivo were classified as moderate irritants).

In summary, determining total protein is not perceived as an essential test in the publications from this literature search. In those instances where good correlations were achieved, other more desireable assays also correlated well, and the test was considered duplicative. When there was poor correlation, other assays were better. In addition, the SDA study highlighted several disadvantages of the TP assay: (1) results obtained from the TP assay depend heavily on the alkalinity of the test sample, and (2) discrimination among extremely irritating substances is poor. Therefore, the TP assay is not considered to be an essential ingredient of an eye irritation screening battery.

<u>Uridine uptake</u>: Test substances are evaluated in this assay by their ability to inhibit radiolabeled uridine uptake by cells in culture. Toxic agents that produce membrane disruption, growth stasis or impair metabolism reduce the rate of uridine uptake. The assay is usually performed by exposing cell cultures to test agents for four hours, after which the cells are washed and incubated for 15 minutes with radiolabeled uridine. After a further wash, followed by lysing, uridine uptake is measured and a protein analysis is performed.

Nineteen materials used in the chemical and cosmetic industry were compared for potential ocular irritation in Balb/c3T3 cells, using the macrophage migration assay, the uridine uptake inhibition assay (UIA) and cytological evaluation as endpoints (Shopsis, et al., 1985). Results obtained from the UIA correlated well with Draize eye irritation data when surfactants and other chemicals were ranked separately. Surfactants were found to be substantially more cytotoxic than the other materials. UIA compared well with the cytological evaluation of the cells and it was claimed that the UIA also has the ability to measure recovery.

The UIA was one of the parameters investigated in the SDA alternatives evaluation program. In Phase I of this program, the assay neither identified correctly the nonirritating agents, but it also failed to correlate significantly with Draize eye irritation scores (Booman et al., 1988). In the second phase of the evaluation, the assay correctly identified only 13/15 FHSA irritants and 7/8 nonirritants and was therefore dropped from further evaluation (Booman et al., 1989).

Data from the few studies reviewed indicate that substantial development needs to occur before this assay can be used routinely. There was much variation between results, and the false negatives and positives achieved in the SDA study mitigate against using the UIA in any

routine screen at the current time.

Primary Cell Culture: As stated previously, the specific cells used in mammalian cell culture test systems can be derived either from established culture cell lines or as primary cultures from selected cells obtained directly from in vivo sources. Theoretically, the use of direct cultures of target cells is preferred because questions of relevancy and sensitivity can be sidestepped. In this review, most investigators prefer to use the established cell lines because it is much easier, the cells are stable and the particular characteristics of the chosen line are well documented. However, as pointed out by Goldberg and Silber (1992), cells in culture often express very different morphology and physiology from the respective in vivo cells. The relatively few researchers who used primary cell cultures either used them as comparisons to established cell lines (Shadduck et al., 1985) or made extensive efforts to ensure, via morphology and antibody staining methods, that the original cell characteristics had not been changed in culture (Grant et al., 1992).

It is not the the purpose of this review to differentiate or extensively classify the results and the references according to whether or not primary or established cell cultures were used by the investigators. Therefore, detailed summaries have not been compiled from references in which primary cell cultures were used, except as they are listed in the section on mammalian cell culture models. Nevertheless, some general comments and conclusions can be made regarding primary cell culture systems as they pertain to the in vitro testing of ocular irritants: (1) changes in enzyme activities in primary cell cultures may be different from those observed in established cell lines (Grant et al., 1992); (2) enzyme levels in cultured cells may decrease with time from those observed in vivo (Grant et al., 1992); and (3) similar rankings of chemicals are generally obtained regardless of cell type, or whether the culture is of primary, or established cell culture type (Borenfreund and Borrero, 1984; Grant et al., 1992; Shadduck et al., 1985).

RBC Lysis/Methemoglobin formation: Bovine, rabbit or rat erythrocytes are used to evaluate potential eye irritation of chemicals and formulations in this assay. The methodology may differ from one investigator to another but the test itself is based on the potential of a chemical to disrupt cell membranes. Formation of methemoglobin (% induction of methemoglobin from hemoglobin), hemolysis (H_{50}), and the denaturation of oxyhemoglobin have all been used as endpoints in the assay.

Hemolysis of bovine erythrocytes in isotonic buffer was used by Muir et al. (1983) to evaluate the ocular irritation potential of eight surfactants; the results of the assay did not correlate well with in vivo rankings. Pape et al. (1987) and Pape and Hoppe (1990) also used bovine erythrocytes to assess the irritancy potential of a large number of ionic and nonionic surfactants (exposure for 10 minutes). These investigators not only used the hemolysis endpoint but also measured the amount of denaturation of oxyhemoglobin that was produced by the various surfactants. Although there were some inconsistencies, on the whole, surfactants with potent hemolytic effects invariably

produced some degree of ocular irritancy in the Draize eye irritation test. Results from denaturation indices and hemolysis tended to parallel each other. Most important of all, the ranking of compounds by all three in vitro methods correlated significantly ($rs = > 0.80$ and $p < 0.0001$) with in vivo Draize eye irritation rankings.

Lysis and methemoglobin induction were also determined in rat erythrocytes. The two in vitro parameters were found to be 77.6% predictive of the in vivo classification for 116 chemicals tested (Sugai et al., 1991b). The non- or mild irritants induced neither methemoglobin formation nor hemolysis; most moderate or severe irritants produced low degrees of hemolysis, and severe irritants had a high probability of inducing methemoglobin formation. Poor correlation was obtained between hemolytic activity and in vivo eye irritation. Poor correlation was also obtained between methemoglobin induction and hemolysis, indicating that these parameters are independent of each other and may reflect different biological endpoints. Cationic and anionic detergents caused both methemoglobin formation and hemolysis. Nonionic detergents produced only hemolytic activity.

The (rabbit) erythrocyte lysis assay was compared to another in vitro assay, K562 cell culture (viability), and to historical Draize eye irritation test scores in a study conducted by Lewis et al., 1993). The RBCs were exposed to 10^{-2} and 10^{-6}M concentrations of 14 selected surfactants. Results indicated that the erythrocyte lysis assay was more predictive of the in vivo results than the cell culture assay. The test correctly identified all irritants and non-irritants, and the ability to rank irritant effects was 89%. The authors concluded that this assay would be a powerful addition to any ocular irritation screen.

A clear consensus about the usefulness of the erythrocyte lysis/methemoglobin formation assay cannot be obtained by reviewing the references in the present literature search. However, generally better results have been reported in the more recent references, and it may be that modifications in technique can account for the differences described in this review. Divergent opinions may also be due to variations in the quality or type of in vivo data used for comparison. It will be of great interest to review the results obtained with this particular assay in Phase III of the CTFA evaluation program. The assay has been proposed as one of eight tests to be evaluated in a rigorous interlaboratory validation program (Gettings, 1993).

SAR/ Physicochemical modeling: Although structure-activity relationship modeling efforts have increased in biological areas, especially in the pharmaceutical and biotechnology industries, relatively little effort has been made to apply this technique to safety testing. The predictive power of the presently available systems are limited to selected classes of chemicals and the inability to predict the physiological effects of complex formulations has been a major limitation in the past (Goldberg and Silber, 1992). According to the PMA Drug Safety Subsection In Vitro Toxicology Task Force (1990), a statistically predictive model for eye irritation has been described, based on a data set of 1,100 chemicals tested in rabbit eyes. Nevertheless, the Task Force rated the SAR models as "inadequate" due to the limited database presently available and

because of the inadequate predictive power for novel compounds.

That the toxicity of surfactants can relate to their molecular structure is not new. Ernst and Arditti (1980) reported, in their evaluation of 13 non-ionic (fatty alcohol ethoxylates) and six amphoteric (sulfobetaines) surfactants, that the physical properties of surfactants were the principal cause of the toxic effects on HeLa cells. In addition, there was a striking correlation between surface tension reduction and toxicity and increasing the lipophilic alkyl chain length of the amphoteric sulfobetaines over a range of 8 to 18 carbon atoms markedly increased this toxicity.

Two more recent SAR-related reports were also found in this literature search. Sugai et al. (1990) studied the relationship of structure to eye irritation in 135 diverse chemicals (medicinals, pesticides, detergents and organic solvents). Thirty-six descriptors were used to describe each molecule and a quantitative structure-activity relationship (QSAR) was then formulated by the adaptive least-squares method to correlate the descriptors with eye irritation. Eighteen descriptors were used to classify each chemical into one of three irritation categories (eye damage which recovered < 24 hours; between 24 hours and 21 days; did not recover by 21 days). All chemicals were applied full strength to the eye. An accuracy of 86.3% was attained in classifying the chemicals correctly into the three categories. It was concluded that this QSAR method was valuable in identifying potential primary ocular irritants.

The semi-quantitative eye irritation potential of 186 organic chemicals was determined by Ptchelintsev et al. (1993) using the Computer Automated Structure Evaluation for Toxicology (CaseTox) model. Three structural descriptors were used to successfully predict the irritation potential of 21 chemicals, mixtures such as coconut oil, eucalyptus oils and potassium alginate, and polymers such as polyvinyl alcohols and polyvinyl pyrrolidone. It was suggested that the model could be used for an in vitro prescreen for eye irritation and also to design and modify chemicals to reduce potential irritation.

Based on the references cited, SAR methodology at present appears to have a limited scope. Until the database for eye irritation is greatly expanded, its use for anything but a rough prescreening tool does not seem to be warranted.

Silicon Microphysiometer: The silicon microphysiometer is a light-addressable potentiometric biosensor that relies on silicon chip technology to indirectly measure metabolic rate by monitoring the rate at which cells excrete their acidic waste products. The detailed methodology is well described by Catroux et al. (1993b) but essentially includes placing confluent culture cells inside the flow chamber of the silicon microphysiometer, determining the baseline metabolic rate of the cells before and directly after exposure to test substance (usually 300-400 seconds) and then calculating the concentration of test material that will decrease the metabolic rate by 50%. This MR_{50} is then used to make comparisons to other test methods.

Rougier et al. (1992) used 41 surfactants, lotions and shampoos to compare six to eight potential in vitro alternatives to the Draize eye irritation test. The silicon microphysiometer was used to establish MR_{50} values in mouse fibroblasts (L 929 cell line) after 400 second exposures to test compounds. Results from this assay provided excellent correlations with Draize MAS (rs = 0.91 for cosmetic products; 0.81 for surfactants; and 0.89 for all 41 products). The data indicated that the silicon microphysiometer gave the highest correlation coefficient with in vivo data of any cell culture procedures used in the study.

Surfactants were also the basic test agents used for evaluation of the silicon microphysiometer in four other references. Catroux et al. (1993a) used a silicon microphysiometer to monitor the physiological state of cultured L929 murine fibroblasts exposed to 19 surfactants and 17 water-soluble surfactant-based formulations. A strong correlation was observed between Draize MAS and the MRD_{50} determinations ($r = 0.89$). The study was later expanded (Catroux et al., 1993b) to include determinations of metabolic effects for 53 products (21 surfactants and 32 surfactant-based formulations). The L929 cells were exposed for 400 seconds to the test substances (10% concentrations for surfactants; neat for formulations). The high correlation between the Draize MAS and the log MR_{50} doses remained unchanged (Spearman correlation coefficient of 0.89). Furthermore, the authors point out that, contrary to other culture cell tests (which can only distinguish two categories of compounds), the silicon microphysiometer makes it possible to classify test agents into three different categories, which roughly correspond to strong, moderate and non- or very weak irritants. Excellent results and correlations with LVET MAS (correlation coefficients of 0.86, $p < 0.0001$ and -0.87) were likewise achieved by Bruner et al. (1991a; 1991b) in the evaluation of 17 surfactant-based materials. It did not seem to make any difference that NHEK cells were used in this case, rather than the murine L929 cells used by Catroux. The authors recommended this assay for screening batteries to detect ocular irritants.

In summary, there is general consensus that the silicon microphysiometer is a good in vitro screening tool for evaluating potential ocular irritants. The assay has produced consistently high correlations with in vivo results, although it must be borne in mind that a rather narrow range of chemical structures was tested. It will be interesting to see if results of the CTFA evaluation (Gettings, 1993) will confirm the results cited in this review. Nevertheless, the assay offers some unique advantages: (1) the short exposure time is more consistent with human accidental exposure and therefore more relevant as a safety assessment procedure; (2) the endpoint reflects a changed physiological state, rather than the most commonly tested parameter, which is cell death. Therefore, the method should be sensitive to products that affect specific metabolic or enzymatic systems short of death; and (3) the silicon microphysiometer may be useful in assessing reversibility because multiple measurements can be made over a defined period of time (Goldberg and Silber, 1992).

Three dimensional models: According to McCulley and Stephens (1993), three dimensional skin models provide a new category of in vitro tests that allow the measurement of multiple endpoints on the same tissue. The models are composed of dermal keratinocytes and fibroblasts

surrounded by a supporting matrix. Test substances can be administered directly to the surface of the preparation, and damage can be evaluated by various endpoints and related to either the length of exposure or to the concentration of the test agent. Proprietary test kits, containing a multilayered cell culture of human fibroblasts and/or keratinocytes and air interphases, are commercially available (Goldberg and Silber, 1992). Such systems have a number of distinct advantages: (1) test substances can be administered directly to the cell surface, thus duplicating actual use conditions and eliminating the confounding effects of solvent vehicles; (2) the primary human cell cultures reflect actual target cells to some extent; and (3) damage can be evaluated by morphological and chemical endpoints as well as by measurement of products of metabolism or inflammation. Whether all of the potential benefits will ever be realized remains to be seen.

Even though the three dimensional models are relatively new assays that have been validated only in a cursory fashion, the systems already have been incorporated into Phase III protocols of the large interlaboratory alternatives evaluation programs sponsored by CTFA and SDA (Gettings, 1993; Bagley et al., in press). The results from the CTFA study have not yet been published, but findings from the Phase III SDA study indicate that the Living Dermal Model correctly classified all 22 test samples according to FHSA criteria, while The Living Skin Equivalent Model misclassified one of the irritants (Bagley et al., in press). Correlation with in vivo Draize MAS was affected by the alkalinity of the test substances in both of these models; The Living Skin Equivalent was much more susceptible (respective correlation coefficients for the Living Dermal Model and The Living Skin Equivalent were = -0.79 and -0.58 when all test substances were considered, and -0.89 and -0.78 when only non-alkaline test materials were included in the calculations). It was also observed that some of the nonirritants exceeded the detection limits of the systems while at the other range of the spectrum, neither system could distinguish (rank) the irritants.

Based on the limited number of references reviewed, the information indicates that the three dimensional models have good theoretical and practical potentials for becoming extremely useful in vitro assays for ocular irritation. The results from the SDA Phase III study are encouraging but at the same time document the fact that there may be substantial differences between variants of the system, and that these assays are subject to some of the same drawbacks as other cell culture systems, e.g. the alkalinity of the test sample.

Wound Closure: Virtually all in vitro assays for ocular irritation are designed to measure acute toxic reactions; whether repair or recovery occurs after removal of the irritant is usually not addressed. Only two assays, the silicon microphysiometer and the wound closure system designed by Jumblatt and coworkers, are considered to be potentially useful for assessing this critical parameter (Goldberg and Silber, 1992). Because the advantages and disadvantages of the silicon microphysiometer have already been addressed in a previous section, only the latter method will be discussed in this section.

The wound closure assay (as originally described by Jumblatt and Neufeld in 1985) has been evaluated by the PMA Drug Safety Subsection In Vitro Toxicology Task Force (1990) and found

to be "inadequate" according to their criteria because the assay is potentially relevant only to the repair mechanism, but not to injury. The method includes establishing a primary cell culture of rabbit corneal epithelial cells (seven days), after which the confluent multilayers are put in suspension, counted and subcultured in culture wells for another week. The confluent subculture in each well is then "wounded" by crushing, then freezing and removing the plug of injured cells beneath a 7mm-diameter filter disc. The remainder of the cell monolayer is then exposed to one of several concentrations of test substance for an appropriate period of time (usually 24 hours), after which the size of the wound area is measured to determine the amount of "healing" that has occurred.

Thirteen test substances (including surfactants, alcohols and preservatives) were evaluated by Simmons et al. (1987) for their relative ability to inhibit wound closure (healing). Dimethyl sulfoxide, ethylene glycol, ethanol, Tween 80, triethanolamine and hydrogen peroxide, all of which are considered to be non- or moderate irritants in the human eye, were also classified as least irritating in the wound closure model. However, the author concludes that it is difficult to directly compare the permissive concentrations of various agents as determined by the wound closure model to literature citations of ocular irritancy. Furthermore, the assay may underestimate the irritating effects of agents, e.g. strong alkalies, which act on other ocular target sites.

Despite the fact that the Jumblatt wound closure assay has not as yet been adequately validated, it was used in Phase I of the SDA alternatives evaluation program, in which eight soap/detergent products were tested by various methodologies. The assay could not differentiate nonirritants from other test compounds, but test results were found to be significantly correlated ($p = < 0.1$) with Draize MAS. The assay was not used in Phase II or III of the study.

Although the wound closure assay is intriguing and one of only two methods that could be used to evaluate recovery potential, the methodology is laborious, requires the sacrifice of animals (if modifications are not made) and assesses only repair, not injury. Furthermore, correlation to in vivo ocular irritation is tenuous, especially in the absence of a substantial database. Therefore, the reviewer concludes that this assay presently is not one that warrants inclusion in an in vitro battery for screening potential ocular irritants.

Discussion and Conclusions

The present literature search reveals that a high level of activity continues to be exerted by scientists from academia, industry and regulatory agencies to attempt to replace the Draize eye irritation test with an appropriate in vitro procedure. Substantial progress has occurred since the publication of the extensive review by Frazier et al. (1987). Excellent overviews by Goldberg and Silber (1992) and McCulley and Stephens (1993) document the progress, and a comprehensive review of the various in vitro proceedures has been produced by the PMA Drug Safety Subsection In Vitro Toxicology Task Force (1990).

It is abundantly clear from a review of the cited publications that finding an appropriate in vitro test system to replace a long-standing in vivo assay such as the Draize eye irritation test is an arduous task, the magnitude of which is hard to comprehend by anyone not familiar with the safety evaluation process and its various regulatory aspects and consumer implications. Not only must an appropriate assay be found, but the procedure must be validated to include many chemical classes, while investigators with a wide range of expertise must be able to reproduce identical results. Each of these phases of the process was well represented in the literature search. In addition, the dedication and collaboration devoted to the goal of replacing the Draize eye irritation test is clearly evident when the review is considered in its entirety.

The contributions of individual investigators, either working in academia or an industrial research laboratory, are evident in the many publications that describe the host of assay modifications or comparison studies, using various chemical entities. These studies are extremely important to the Draize replacement project because they expand the available data base and replicate valid results that may have been questionable. Results from a series of such studies can then be used as the basis for scientific conclusions.

The contributions of industry are also quite apparent in the review. Results currently emerging from the large industry-sponsored interlaboratory validation studies will have a great impact on the ultimate selection of in vitro assays that can be used successfully as screening tools to evaluate potential eye irritants and the speed with which these assays become operational.

Regulatory agencies, from the United States as well as from the EC, have also contributed to the overall effort. Their participation at various international, interagency and industry/government meetings is extremely productive because sound regulatory decisions cannot be made in a void, and regulatory agency expectations must be known and then broadly discussed in a scientific forum. On the domestic scene, participation in meetings such as the Joint Government-Industry Workshop on Progress Towards Non-Animal Alternatives to the Draize Test, held in Arlington, Virginia on September 14, 1988 (Frazier, 1989) and the interagency Workshop on Updating Eye Irritation Test Methods: Proposals for Regulatory Consensus, held in Washington, D.C. on September 26-27, 1991 (Green et al., 1993) have been immensely helpful in gaining a consensus for in vitro testing program requirements.

Meetings and dissemination of information has also occurred on the international level. In October of 1992, a joint UK/EC-sponsored meeting on alternatives, "An International Validation Study on Alternatives to the Draize Eye Irritation Test for the Classification and Labeling of Chemicals," was held in London (Wilcox, 1992). The purpose of the conference was to evaluate an international , multilaboratory study of in vitro assays for detecting ocular irritation. Such endeavors are necessary not only to reach a consensus on testing requirements, but to harmonize, wherever possible, the regulations that will follow any new methodologies.

Was it possible to draw any conclusions from the literature search regarding these methodologies? Several general, as well as some specific, impressions were gained from a review of the

publications. First, the most prevalent finding was that there is a wide divergence of opinion as to which assays, if any, are the best predictors of potential eye irritants. This difference of opinion usually was based on the experimental results achieved by individual investigators, and the reason for the difference in results often was not clear. For example, Decker and Harper (1993) reported that the correlation between Draize and EYTEX irritation classifications were 100% and 87% for adult and baby shampoos, respectively. They concluded that the EYTEX system was highly predictive of maximum 24-hour Draize eye irritation scores. On the other hand, Bruner et al. (1991a) obtained completely random relationships between in vivo and EYEX results when they tested 17 surfactant-based chemicals and consumer products. Such differences in the data were not uncommon in this literature search; frequently the differences could be attributed to variations in types of chemicals tested. This concept was stated by many authors and leads to the second general impression: the in vitro battery of tests that will most accurately predict ocular irritation will most probably have to be tailored to the class of compounds to be tested. Only time will reveal whether this impression is correct.

No single in vitro assay is considered to be capable of replacing the Draize eye irritation test, a point that has been made consistently in most of the publications. Therefore, which assays should be selected for an in vitro irritation battery? The answer, based on the material included in this review, must unfortunately be somewhat subjective because it is influenced to a great degree by the number, type and quality of the publications reviewed. However, the CAM or HET-CAM assay, the chicken EET, the neutral red and plasminogen activation assays for cytotoxicity, and the silicaon microphysiometer appear to have the greatest potential as possible screening tools for an eye irritation screen. These assays represent a variety of endpoints and reflect the prevalent opinion that mechanistic variation should be considered in the selection process. No distinction has been made as to whether the tests should be used as a screening procedures to decrease in vivo testing, or as a replacement for the entire Draize eye irritation test.

How these assays will be applied in safety testing programs, and in what combinations the tests will be used has yet to be determined. Various tier testing programs have been proposed, including those mentioned by Atkinson et al. (1992), Frazier (1989), Gupta et al. (1993), McCormick (1989) and PMA (1990). Further testing and validation are ongoing, and it is hoped that results from the large-scale interlaboratory trials will help determine the testing schema of the near future. An accurate summary of the present state of the art was given by Scala (1993) when he stated that, "The Draize eye irritation test will not yet pass into disuse; however, alternatives have now attained the status of screening tests. Agents that 'fail' in such screens are not candidates for animal tests, but agents that 'pass' may require in vivo testing before being introduced to widespread human exposure".

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APPENDIX

Table 2:	Listing of Selected Chemicals and Formulations Tested *In Vitro*
Table 3:	List of *In Vitro/In Vivo* Correlations

Table 2
Listing of Selected Chemicals and Formulations Tested *In Vitro*

Chemical	Concentration*	Endpoint	Reference
Acylamine polyglycol ethersulfate	not reported	AD;BEET;EYTEX;HET-CAM;LB;NR;Prot;SM	Rougier (1992)
Alcohol ethoxylate	10%	prot;TT; CAM/VA;PA;cytotox;dermal;NR	Bagley (in press)
Alkanesulfonate	1%; increasing conc.	Bovine RBC hemolysis/denaturation	Pape (1990)
Alkyl benzyl dimethyl ammonium chloride	0.50%	colony formation (4 cell lines)	Sasaki (1991)
Alkyl-N-carboxymethyl-N-hydroxyethyl imidaxolinium betaine	0.50%	colony formation (4 cell lines)	Sasaki (1991)
Alkylbenzenesulfonate	1%; increasing conc.	Bovine RBC hemolysis/denaturation	Pape (1990)
Alkylethoxylated sulfates (2)	serial 2-fold solutions	Chromium release	Shadduck (1995)
Alkylethoxysulfate	10%	prot;TT; CAM/VA;PA;cytotox;dermal;NR	Bagley (in press)
Alkylglyceryl sulfate	10%	prot;TT; CAM/VA;PA;cytotox;dermal;NR	Bagley (in press)
Allyl alcohol	5-10%	BEET (opacity/thickness)	Igarashi (1987)
Ammonium dodecylsulfate	not reported	AD;BEET;EYTEX;HET-CAM;LB;NR;Prot;SM	Rougier (1992)
Ammonium laureth-2-sulfate	1%; increasing conc.	Bovine RBC hemolysis/denaturation	Pape (1990)
Ammonium lauryl sulfate	40ug/ml - 200 mg/ml	Silicon Microphysiometer	Catroux (1993b)
Ammonium lauryl sulfate	10%	prot;TT; CAM/VA;PA;cytotox;dermal;NR	Bagley (in press)
Ammonium lauryl sulfate	1%; increasing conc.	Bovine RBC hemolysis/denaturation	Pape (1990)
Baby shampoo US brand	100%	BEET (opacity/permeability)	Vanparys (1993)
Benzalconium chloride	40, 60, 80, 100%	BEET (opacity/permeability)	Stankowski (1993)
Benzalconium chloride	serial 2-fold solutions	Chromium release	Shadduck (1995)
Benzalkonium chloride	<0.006mM	MTT/dye reduction/NR/prot	Sina (1992)
Benzalkonium chloride	not reported	RBC hemolysis/Hb formation	Sugai (1991b)
Benzalkonium chloride	0.01%	wound closure	Simmons (1987)
Benzalkonium chloride	0.8 ug/ml	uridine uptake (HTD)	Shopsis (1985)
Benzalkonium chloride	10,5,1%	growth inhibition (cytotox)	Kennah (1989a)
Benzalkonium chloride	0.001-10mg/ml	SM; LB; NR; Prot; TT; BE/CAM; EYTEX	Bruner (1991a)
Benzalkonium chloride	<6 ug/ml	NR; fluorescein; cell detachment, growth inhibition	Bracher (1987)
Benzalkonium chloride	0.003 mM	HTD (cytotox); ID50 (colony formation)	Borenfreund (1984)
Benzalkonium chloride	1%; increasing conc.	Bovine RBC hemolysis/denaturation	Pape (1990)
Benzalkonium chloride	10%	Eytex	Gordon (1992)
Benzalkonium chloride	5 & 10%	LDH leakage;NR;MTT;morphology (cytotox)	Grant (1992)
Benzalkonium chloride (10% sol.)	1.8 ug/ml	MTT dye reduction (viability IC50)	Nagami (1993)
Benzethonium chloride	0.50%	colony formation (4 cell lines)	Sasaki (1991)
Betaine	1%	Eytex	Gordon (1992)
Boric acid	neat (?)	Eytex	Gordon (1992)
Butanol	100%	BEET (opacity/permeability)	Vanparys (1993)
Butoxyethanol	5%	prot;TT; CAM/VA;PA;cytotox;dermal;NR	Bagley (in press)
Butoxyethanol	20,40,60,80,100%	BEET (opacity/permeability)	Gautheron (1992)
Butyl alcohol	11.1 mg/ml	MTT dye reduction (viability IC50)	Nagami (1993)
Cetyl trimethylammonium bromide	0.05M in vivo	fluorescein/EB dye uptake; alk phos release (cytotox)	Scaife (1985)
Cetyl trimethylammonium bromide	.01-.0001M	BEET (opacity)	Muir (1985)
Citric acid	neat (?)	Eytex	Gordon (1992)
Cocoamidopropylbetaine	1%; increasing conc.	Bovine RBC hemolysis/denaturation	Pape (1990)
Cocoamphocarboxyglycinate	1%; increasing conc.	Bovine RBC hemolysis/denaturation	Pape (1990)
Cocobetain derivative	not reported	AD;BEET;EYTEX;HET-CAM;LB;NR;Prot;SM	Rougier (1992)
Cocodimethylaminoxide	1%; increasing conc.	Bovine RBC hemolysis/denaturation	Pape (1990)
Cocodimethylbetaine	1%; increasing conc.	Bovine RBC hemolysis/denaturation	Pape (1990)
Coconut fatty acid diethanolamide	0.50%	colony formation (4 cell lines)	Sasaki (1991)
Decyl polyglucoside	0.01-0.000001 M	rabbit RBC hemolysis;cell viability	Lewis (1993)
Dimethyl distearyl ammonium chloride	0.50%	colony formation (4 cell lines)	Sasaki (1991)
Dimethylditallowammonium chloride	3%	prot;TT; CAM/VA;PA;cytotox;dermal;NR	Bagley (in press)

*If percent of active ingredient was not stated, the best representative dose (e.g. IC50) is given.

Table 2

Listing of Selected Chemicals and Formulations Tested *In Vitro*

Chemical	Concentration*	Endpoint	Reference
Dioxane	5-10%	BEET (opacity/thickness)	Igarashi (1987)
Distearyl dimethyl ammonium chloride	0.50%	colony formation (4 cell lines)	Sasaki (1991)
Dodecanedio (etherified)	not reported	AD;BEET;EYTEX;HET-CAM;LB;NR;Prot;SM	Rougier (1992)
Dodecanol (etherified)	not reported	AD;BEET;EYTEX;HET-CAM;LB;NR;Prot;SM	Rougier (1992)
Dodecylamine oxide	1%	prot;TT; CAM/VA;PA;cytotox;dermal;NR	Bagley (in press)
Ethanol	65.0 mg/ml	MTT dye reduction (viability IC50)	Nagami (1993)
Ethanol	5-10%	BEET (opacity/thickness)	Igarashi (1987)
Ethanol	<865 mM	MTT/dye reduction/NR/prot	Sina (1992)
Ethanol	.058-0.58%	wound closure	Simmons (1987)
Ethanol	500 mM	uridine uptake (HTD)	Shopsis (1985)
Ethanol	15%	prot;TT; CAM/VA;PA;cytotox;dermal;NR	Bagley (in press)
Ethanol	40,60,80,100%	BEET (opacity/permeability)	Gautheron (1992)
Ethanol	95%	REET (corneal thickness)	Burton (1981)
Ethanol	342-513 mM	HTD (cytotox); ID50 (colony formation)	Borenfreund (1984)
Ethanol	0.50%	colony formation (4 cell lines)	Sasaki (1991)
Ethanol	neat (?)	Eytex	Gordon (1992)
Ethanol	40, 60, 80, 100%	BEET (opacity/permeability)	Stankowski (1993)
Ethylene glycol	92.2 mg/ml	MTT dye reduction (viability IC50)	Nagami (1993)
Ethylene glycol	40,60,80,100%	BEET (opacity/permeability)	Gautheron (1992)
Ethylene glycol	89-253 mM	HTD (cytotox); ID50 (colony formation)	Borenfreund (1984)
Ethylene glycol	40, 60, 80, 100%	BEET (opacity/permeability)	Stankowski (1993)
Glycerine	neat	REET (corneal thickness)	Burton (1981)
Glycerine	0.50%	colony formation (4 cell lines)	Sasaki (1991)
Glycerol	84.3 mg/ml	MTT dye reduction (viability IC50)	Nagami (1993)
Glycerol	neat	prot;TT; CAM/VA;PA;cytotox;dermal;NR	Bagley (in press)
Glycerol	neat (?)	Eytex	Gordon (1992)
Hexadecyltrimethylammonium bromide	not reported	AD;BEET;EYTEX;HET-CAM;LB;NR;Prot;SM	Rougier (1992)
Hexane	neat (?)	Eytex	Gordon (1992)
Hexanol	100, 30, 10%	growth inhibition (cytotox)	Kennah (1989a)
Isopropanol	32.6 mg/ml	MTT dye reduction (viability IC50)	Nagami (1993)
Isopropanol	<378mM	MTT/dye reduction/NR/prot	Sina (1992)
Isopropanol	15%	prot;TT; CAM/VA;PA;cytotox;dermal;NR	Bagley (in press)
Isopropanol	40,60,80,100%	BEET (opacity/permeability)	Gautheron (1992)
Isopropanol	104.0 mM	HTD (cytotox); ID50 (colony formation)	Borenfreund (1984)
Isopropanol	100, 30, 10%	growth inhibition (cytotox)	Kennah (1989a)
Isopropanol	40, 60, 80, 100%	BEET (opacity/permeability)	Stankowski (1993)
Laureth-sulphosuccinate	1%; increasing conc.	Bovine RBC hemolysis/denaturation	Pape (1990)
Lauryl dimethylaminoacetic acid betaine	0.50%	colony formation (4 cell lines)	Sasaki (1991)
Lauryl trimethylammonium bromide	0.01 M; 0.001 M	BEET (opacity/thickness)	Igarashi (1987)
Lauryl trimethylammonium bromide	0.05M in vivo	fluorescein/EB dye uptake; alk phos release (cytotox)	Scaife (1985)
Lauryl trimethylammonium bromide	.01-.0001M	BEET (opacity)	Muir (1985)
Linoleic Acid	neat (?)	Eytex	Gordon (1992)
Methoxyethanol	neat (?)	Eytex	Gordon (1992)
Methylparaben	neat (?)	Eytex	Gordon (1992)
Monoethanolamine tallow acid amide	0.50%	colony formation (4 cell lines)	Sasaki (1991)
Myristoyl diethanoleate	0.50%	colony formation (4 cell lines)	Sasaki (1991)
Myristyl trimethylammonium bromide	0.05M in vivo	fluorescein/EB dye uptake; alk phos release (cytotox)	Scaife (1985)
Myristyl trimethylammonium bromide	.01-.0001M	BEET (opacity)	Muir (1985)
Nonoxynol-8	0.01-0.000001 M	rabbit RBC hemolysis;cell viability	Lewis (1993)

*If percent of active ingrediant was not stated, the best representative dose (e.g. IC50) is given.

Table 2
Listing of Selected Chemicals and Formulations Tested *In Vitro*

Chemical	Concentration*	Endpoint	Reference
Octanol	100%	BEET (opacity/permeability)	Vanparys (1993)
Octyl phenoxypolyethoxy ethanol (=Triton X 100)	not reported	AD;BEET;EYTEX;HET-CAM;LB;NR;Prot;SM	Rougier (1992)
Olefinsulfonate	1%; increasing conc.	Bovine RBC hemolysis/denaturation	Pape (1990)
Pentadecanol (etherified)	not reported	AD;BEET;EYTEX;HET-CAM;LB;NR;Prot;SM	Rougier (1992)
Phenoxyethanol	0.50%	colony formation (4 cell lines)	Sasaki (1991)
Polyethylene -24-glycerylsterate	1%; increasing conc.	Bovine RBC hemolysis/denaturation	Pape (1990)
Polyethylene glycol 40 stearate	0.01-0.000001 M	rabbit RBC hemolysis;cell viability	Lewis (1993)
Polyethylene glycol 400	neat	prot;TT; CAM/VA;PA;cytotox;dermal;NR	Bagley (in press)
Polyethylene glycol 400	0.50%	colony formation (4 cell lines)	Sasaki (1991)
Polyethylene-10-nonylphenol	1%; increasing conc.	Bovine RBC hemolysis/denaturation	Pape (1990)
Polyoxyethylene glycol monolaurate (10 E.O.)	0.50%	colony formation (4 cell lines)	Sasaki (1991)
Polyoxyethylene glycol monooleate (25 E.O.)	0.50%	colony formation (4 cell lines)	Sasaki (1991)
Polyoxyethylene lauryl ether	0.50%	colony formation (4 cell lines)	Sasaki (1991)
Polyoxyethylene laurylether phosfate	0.50%	colony formation (4 cell lines)	Sasaki (1991)
Polyoxyethylene sorbitan monolaurate (20 E.O.)	0.50%	colony formation (4 cell lines)	Sasaki (1991)
Polyoxyethylene sorbitan monooleate (20 E.O.)	0.50%	colony formation (4 cell lines)	Sasaki (1991)
Polyoxyethylene sorbitan monooleate (20 E.O.)	0.50%	colony formation (4 cell lines)	Sasaki (1991)
Polyoxyethylene sorbitane monolaurate	not reported	AD;BEET;EYTEX;HET-CAM;LB;NR;Prot;SM	Rougier (1992)
Polyoxyethylene sorbitane monooleate	not reported	AD;BEET;EYTEX;HET-CAM;LB;NR;Prot;SM	Rougier (1992)
Polyoxyethylene sorbitol tetraoleate	0.50%	colony formation (4 cell lines)	Sasaki (1991)
Polysorbate 20	serial 2-fold solutions	Chromium release	Shadduck (1995)
Potassium laurate	0.50%	colony formation (4 cell lines)	Sasaki (1991)
Propylene glycol	6.27 mg/ml	MTT dye reduction (viability IC50)	Nagami (1993)
Propylene glycol	5-10%	BEET (opacity/thickness)	Igarashi (1987)
Propylene glycol	100%	BEET (opacity/permeability)	Vanparys (1993)
Propylene glycol	40,60,80,100%	BEET (opacity/permeability)	Gautheron (1992)
Propylene glycol	neat	REET (corneal thickness)	Burton (1981)
Propylene glycol	435 mM	HTD (cytotox); ID50 (colony formation)	Borenfreund (1984)
Propylene glycol	40, 60, 80, 100%	BEET (opacity/permeability)	Stankowski (1993)
Pyridinium cetylbromide	not reported	AD;BEET;EYTEX;HET-CAM;LB;NR;Prot;SM	Rougier (1992)
Sodium alkyl sulfate	10%	prot;TT; CAM/VA;PA;cytotox;dermal;NR	Bagley (in press)
Sodium cetyl sulfate	3% in vivo	fluorescein/EB dye uptake; alk phos release (cytotox)	Scaife (1985)
Sodium coconut fatty acid taurate	0.50%	colony formation (4 cell lines)	Sasaki (1991)
Sodium decyl sulfate	3% in vivo	fluorescein/EB dye uptake; alk phos release (cytotox)	Scaife (1985)
Sodium decyl sulfate	.01-.0001M	BEET (opacity)	Muir (1985)
Sodium dodecyl sulfate	<3.5mM	MTT/dye reduction/NR/prot	Sina (1992)
Sodium dodecyl sulfate	not reported	RBC hemolysis/Hb formation	Sugai (1991b)
Sodium dodecyl sulfate	30,15,3%	growth inhibition (cytotox)	Kennah (1989a)
Sodium dodecyl sulfate	<197 ug/ml	NR; fluorescein; cell detachment, growth inhibition	Bracher (1987)
Sodium dodecyl sulfate	0.28 mM	HTD (cytotox); ID50 (colony formation)	Borenfreund (1984)
Sodium dodecyl sulfate	serial 2-fold solutions	Chromium release	Shadduck (1995)
Sodium dodecyl sulfate (SDS)	not reported	AD;BEET;EYTEX;HET-CAM;LB;NR;Prot;SM	Rougier (1992)
Sodium dodecylethersulfate	not reported	AD;BEET;EYTEX;HET-CAM;LB;NR;Prot;SM	Rougier (1992)
Sodium hydroxide	not reported	RBC hemolysis/Hb formation	Sugai (1991b)
Sodium hydroxide	0.01%	wound closure	Simmons (1987)
Sodium hydroxide	1N	corneal thickness and morphology (rabbit/human)	Berry (1993)
Sodium hypochlorite	1mM	uridine uptake (HTD)	Shopsis (1985)
Sodium hypochlorite	5.25%	prot;TT; CAM/VA;PA;cytotox;dermal;NR	Bagley (in press)
Sodium hypochlorite	0.28-2.13 mM	HTD (cytotox); ID50 (colony formation)	Borenfreund (1984)

*If percent of active ingredient was not stated, the best representative dose (e.g. IC50) is given.

Table 2

Listing of Selected Chemicals and Formulations Tested *In Vitro*

Chemical	Concentration*	Endpoint	Reference
Sodium Lauryl N methyglycinate	not reported	AD;BEET;EYTEX;HET-CAM;LB;NR;Prot;SM	Rougier (1992)
Sodium lauryl sarcosinate	40ug/ml - 200 mg/ml	Silicon Microphysiometer	Catroux (1993b)
Sodium lauryl sulfate	11.7 ug/ml	MTT dye reduction (viability IC50)	Nagami (1993)
Sodium lauryl sulfate	40ug/ml - 200 mg/ml	Silicon Microphysiometer	Catroux (1993b)
Sodium lauryl sulfate	0.01 M; 0.001 M	BEET (opacity/thickness)	Igarashi (1987)
Sodium lauryl sulfate	85 ug/ml	uridine uptake (HTD)	Shopsis (1985)
Sodium lauryl sulfate	3% in vivo	fluorescein/EB dye uptake; alk phos release (cytotox)	Scaife (1985)
Sodium lauryl sulfate	10%	prot;TT; CAM/VA;PA;cytotox;dermal;NR	Bagley (in press)
Sodium lauryl sulfate	0.001-10mg/ml	SM; LB; NR; Prot; TT; BE/CAM; EYTEX	Bruner (1991a)
Sodium lauryl sulfate	0.50%	colony formation (4 cell lines)	Sasaki (1991)
Sodium lauryl sulfate	1%; increasing conc.	Bovine RBC hemolysis/denaturation	Pape (1990)
Sodium lauryl sulfate	2%	corneal thickness and morphology (rabbit/human)	Berry (1993)
Sodium lauryl sulfate	.01-.0001M	BEET (opacity)	Muir (1985)
Sodium lauryl sulfate (SDS)-B	40ug/ml - 200 mg/ml	Silicon Microphysiometer	Catroux (1993b)
Sodium perborate, powder	5.36%	prot;TT; CAM/VA;PA;cytotox;dermal;NR	Bagley (in press)
Sodium polyethylene lauryl ether sulfate (12 E.O.)	0.50%	colony formation (4 cell lines)	Sasaki (1991)
Sodium tetradecenesulfonate	0.50%	colony formation (4 cell lines)	Sasaki (1991)
Sodium-laureth-2-sulfate	1%; increasing conc.	Bovine RBC hemolysis/denaturation	Pape (1990)
Sodium-soap (unbuff.)	1%; increasing conc.	Bovine RBC hemolysis/denaturation	Pape (1990)
Sorbitan monooleate	0.50%	colony formation (4 cell lines)	Sasaki (1991)
Steareth-100	0.01-0.000001 M	rabbit RBC hemolysis;cell viability	Lewis (1993)
Stearyl dihydroxyethoxyethyl hydroxyethyl ammonium chloride	0.50%	colony formation (4 cell lines)	Sasaki (1991)
Stearyl trimethyl ammonium chloride	0.50%	colony formation (4 cell lines)	Sasaki (1991)
Sucrose fatty acid ester	0.50%	colony formation (4 cell lines)	Sasaki (1991)
Sucrose laurinic acid ester	0.50%	colony formation (4 cell lines)	Sasaki (1991)
TEA-dodecanoate	1%; increasing conc.	Bovine RBC hemolysis/denaturation	Pape (1990)
TEA-laureth-2-sulfate	1%; increasing conc.	Bovine RBC hemolysis/denaturation	Pape (1990)
TEA-laurylsulfate	1%; increasing conc.	Bovine RBC hemolysis/denaturation	Pape (1990)
Tetradecyltrimethylammonium bromide	not reported	AD;BEET;EYTEX;HET-CAM;LB;NR;Prot;SM	Rougier (1992)
Triethanolamine	0.10%	wound closure	Simmons (1987)
Triethanolamine cetyl sulfate	0.1M in vivo	fluorescein/EB dye uptake; alk phos release (cytotox)	Scaife (1985)
Triethanolamine decyl sulfate	0.1M in vivo	fluorescein/EB dye uptake; alk phos release (cytotox)	Scaife (1985)
Triethanolamine decyl sulfate	.01-.0001M	BEET (opacity)	Muir (1985)
Triethanolamine lauryl sulfate	0.1M in vivo	fluorescein/EB dye uptake; alk phos release (cytotox)	Scaife (1985)
Triethanolamine lauryl sulfate	.01-.0001M	BEET (opacity)	Muir (1985)
Triethanolamine myristyl sulfate	0.1M in vivo	fluorescein/EB dye uptake; alk phos release (cytotox)	Scaife (1985)
Triethanolamine myristyl sulfate	.01-.0001M	BEET (opacity)	Muir (1985)
Triethylammonium lauryl sulfate	10%	prot;TT; CAM/VA;PA;cytotox;dermal;NR	Bagley (in press)
Triton X 100	10,5,1%	growth inhibition (cytotox)	Kennah (1989a)
Tween 20	219 ug/ml	MTT dye reduction (viability IC50)	Nagami (1993)
Tween 20	5-10%	BEET (opacity/thickness)	Igarashi (1987)
Tween 20	100%	BEET (opacity/permeability)	Vanparys (1993)
Tween 20	<4.5 mM	MTT/dye reduction/NR/prot	Sina (1992)
Tween 20	10,5,1%	growth inhibition (cytotox)	Kennah (1989a)
Tween 20	40,60,80,100%	BEET (opacity/permeability)	Gautheron (1992)
Tween 20	0.001-10mg/ml	SM; LB; NR; Prot; TT; BE/CAM; EYTEX	Bruner (1991a)
Tween 20	not reported	AD;BEET;EYTEX;HET-CAM;LB;NR;Prot;SM	Rougier (1992)
Tween 20	neat	corneal thickness and morphology (rabbit/human)	Berry (1993)
Tween 20	1,5,10%	LDH leakage	Grant (1992)

*If percent of active ingredient was not stated, the best representative dose (e.g. IC50) is given.

Table 2
Listing of Selected Chemicals and Formulations Tested *In Vitro*

Chemical	Concentration*	Endpoint	Reference
Tween 20	40, 60, 80, 100%	BEET (opacity/permeability)	Stankowski (1993)
Tween 40	227 ug/ml	MTT dye reduction (viability IC50)	Nagami (1993)
Tween 40	190 ug/ml	uridine uptake (HTD)	Shopsis (1985)
Tween 60	230 ug/ml	uridine uptake (HTD)	Shopsis (1985)
Tween 80	270 ug/ml	MTT dye reduction (viability IC50)	Nagami (1993)
Tween 80	5-10%	BEET (opacity/thickness)	Igarashi (1987)
Tween 80	100%	BEET (opacity/permeability)	Vanparys (1993)
Tween 80	0.10%	wound closure	Simmons (1987)
Tween 80	400 ug/ml	uridine uptake (HTD)	Shopsis (1985)

MTT = tetrazolium salt dye
BEET = bovine enucleated eye test
NR = neutral red
RBC = red blood cell
Hb = hemoglobin
HTD = highest tolerated dose
EB = ethidium bromide
alk phos = alkaline phosphatase
prot = protein
CAM/VA = chorioallantoic membrane/vascular assay
PA = plasminogen activation
REET = rabbit enucleated eye test
LB = luminescent bacteria
TT = T. thermophile motility
BE/CAM = bovine eye/chorioallantoic membrane
AD = agar diffusion
HET-CAM = Hen egg test-chorioallantoic membrane
SM = silicon microphysiometer
LDH = lactic dehydrogenase

*If percent of active ingredient was not stated, the best representative dose (e.g. IC50) is given.

Table 3
Listing of *In Vitro*/*In Vivo* Correlations

Agar diffusion (lysis diameter)	Corr. coef. = < 0.5 (in house MAS)	Rougier (1992)
Alk phos release	GR: poor correlation with detergents	Scaife (1985)
BE/CAM (opacity/vascular damage)	Corr. coef. = -0.29 (LVET: Griffith, 1980)	Bruner (1991a)
CAM (HET/CAM)	Corr. coef. = 0.96 (in house MAS)	Rougier (1992)
CAM/VA	Corr. coef. = -0.91 (concurrent Draize MAS)	Bagley (in press)
Cell detachment (25% detachment)	GR: similar (guinea pigs)	Bracher (1987)
Chromium release (50% release)	GR: ranking similar to in-house LVET	Shadduck (1985)
Colony formation (50% inhibition)	GR: good agreement	Borenfreund (1984)
Colony formation (50% inhibition)	Corr. coef. = 0.57-0.71 (4 cell lines) (Watanbe '89)	Sasaki (1991)
Colony formation (50% inhibition)	GR: ranking similar (in-house data)	North-Root (1985)
Cytotoxicity (trypan blue uptake)	ranking corr. = 57% (in-house data)	Lewis (1992)
EET- bovine (opacity)	Corr. coef. = 0.854 with Draize (Muir, 1983)	Muir (1985)
EET- bovine (opacity/permeability)	Corr. coef. = 0.83 with Draize (Gautheron '92)	Stankowski (1993)
EET- bovine (opacity/permeability)	96% concordance Draize ranking (4 gps.) (OECD 1987 data)	Vanparys (1993)
EET- bovine (opacity/permeability)	Corr. coef. = 0.73 (Clayton, '82; Carpenter, '46; Grant, '86; Kennah, '89)	Gautheron (1992)
EET- bovine (opacity/permeability)	Corr. coef. = 0.77 (in house MAS)	Rougier (1992)
EET- bovine (opacity/permeability) cont.	Corr. coef. = 0.73 (Clayton, '82; Carpenter, '46; Grant, '86; Kennah, '89)	Gautheron (1992)
EET- bovine (opacity/thickness)	No comparison made to Draize Test	Igarashi (1987)
EET- rabbit (corneal thickness)	GR: good agreement - 4gps. (in-house data)	Price (1985)
EET- rabbit (opacity/thickness)	GR: agreement for weak & severe irritants (Carpenter, '46)	Burton (1991)
EYTEX	Corr. coef. = 0.74 (in house MAS)	Rougier (1992)
EYTEX (increased matrix opacity)	could not be calculated	Bruner (1991a)
EYTEX (increased matrix opacity)	GR: "equivalence" of 91% with Draize test (unspecified data)	Gordon (1992)
Fluorescein/EB (25% reduced viability)	GR: similar (guinea pigs)	Bracher (1987)
Fluorescein/EB uptake	GR: poor correlation with detergents	Scaife (1985)
Fluorescein/EB uptake(CD50)	GR: good agreement with irritants (in-house data)	Kemp (1983)
Growth inhibition (50% inhibition)	GR: similar (guinea pigs)	Bracher (1987)
Growth inhibition (50% inhibition)	Corr. coef. = 0.56 (in-house Draize data)	Kennah (1989a)
LDH (% leakage)	GR: similar ranking (Kennah, 1989)	Grant (1992)
LDH leakage	Corr. coef. = 0.657 with Draize (no ref.)	Marinovich (1993)
Luminescent bacteria (50% inhibition)	Corr. coef. = 0.79 (in house MAS)	Rougier (1992)
Luminescent bacteria (50% light reduction)	Corr. coef. = -0.91 (LVET: Griffith, 1980)	Bruner (1991a)
Morphology (HTD)	GR: good agreement	Borenfreund (1984)
Morphology (HTD)	no reference to in vivo	Riddell (1986)
Morphology (LC50)	Corr. coef. = -0.61 (concurrent Draize MAS)	Bagley (in press)
MTT (50% reduced viability)	Corr. coef. IC50:DSo = 0.82 (concurrent)	Nagami (1993)
MTT (50% viability)	<-0.5 with Draize (Carpenter & Smythe 1944-74)	Sina (1991)
Neutral red	Corr. coef. = -0.74 to -0.78 (concurrent Draize MAS)	Bagley (in press)
Neutral red (50% inhibition)	<-0.5 with Draize (Carpenter & Smythe 1944-74)	Sina (1991)
Neutral red (50% inhibition)	GR: similar (guinea pigs)	Bracher (1987)

GR=general ranking only

Table 3
Listing of *In Vitro*/*In Vivo* Correlations

Neutral red (50% inhibition)	Corr. coef. = -0.85 (LVET: Griffith, 1980)	Bruner (1991a)
Neutral red (50% inhibition)	no reference to in vivo	Riddell (1986)
Neutral red (50% inhibition)	Corr. coef. = 0.80 (in house MAS)	Rougier (1992)
Plasminogen activitation	Corr. coef. = -0.85 (concurrent Draize MAS)	Bagley (in press)
Protein (50% inhibition)	<-0.5 with Draize (Carpenter & Smythe 1944-74)	Sina (1991)
Protein (50% inhibition)	Corr. coef. = -0.74 (concurrent Draize MAS)	Bagley (in press)
Protein (50% inhibition)	no reference to in vivo	Riddell (1986)
Protein (50% inhibition)	Corr. coef. = 0.56 (in house MAS)	Rougier (1992)
Protein (inhibition IC50)	Corr. coef. = 0.524 with Draize (no ref.)	Marinovich (1993)
RBC hemolysis/denaturation (Bovine)	GR: ranking similar (OECD Guideline)	Pape (1990)
RBC hemolysis/denaturation (Bovine)	Corr. coef. = 0.80 (Kastner, 1981)	Pape (1987)
RBC hemolysis/denaturation (Rabbit)	ranking corr. = 89% (in-house data)	Lewis (1992)
RBC hemolysis/Hb formation (rat)	77.6% concordance Draize ranking (3 gps) (in house data)	Sugai (1991b)
Silicon microphysiometer	Corr. coef. = 0.89 (in house MAS)	Rougier (1992)
Silicon microphysiometer (MRD 50)	Corr. coef. MRD:MAS = 0.89-0.91 (concurrent)	Catroux (1993b)
Silicon microphysiometer (MRD 50)	Corr. coef. = -0.87 (LVET: Griffith, 1980)	Bruner (1991a)
T. thermophila motility	Corr. coef. = 0.85 (concurrent Draize MAS)	Bagley (in press)
T. thermophila motility (slightly reduced motility)	Corr. coef. = 0.78 (LVET: Griffith, 1980)	Bruner (1991a)
Three-D dermal model	Corr. coef. = -0.79 (concurrent Draize MAS)	Bagley (in press)
Three-D skin/MTT (50% dye inhibition)	sensitivity = 83%; specificity = 88%	Reece (1993)
Total protein (50% reduction)	Corr. coef. = -0.86 (LVET: Griffith, 1980)	Bruner (1991a)
Uridine uptake (HTD)	GR: similar ranking (Grant '74; Clayton '82)	Shopsis (1985)
Wound closure	GR: general ranking similar to human (Grant '74)	Simmons (1987)

EET = enucleated eye test
MTT = tetrazolium salt dye
RBC = red blood cell
HTD = highest tolerated dose
CAM/VA = chorioallantoic membrane/vascular assay
EB = ethidium bromide
BE/CAM = bovine eye/chorioallantoic membrane
HET/CAM = hen egg test/chorioallantoic membrane
LDH = lactic dehydrogenase

GR = general ranking only