

## ABSTRACT

**A HISTOPATHOLOGICAL AND CYTOLOGICAL EVALUATION OF BOVINE CORNEAS EXPOSED TO ANIONIC OR CATIONIC SURFACTANTS IN A BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY. D. R. Cerven<sup>1</sup>, L. H. Arp<sup>2</sup>, P. V. Shah<sup>2</sup>, O. M. Moreno<sup>1</sup>. <sup>1</sup>MB research Lab, Spinnerstown, PA; <sup>2</sup>Hoffmann-La Roche, Inc., Nutley, NJ**

In this study, we determined the histological differences of bovine corneas after exposure to two types of surfactants. Bovine corneas were exposed to a 10% aqueous solution of dioctyl sulfo-succinate (DOSS) or a 0.5% aqueous solution of Benzalkonium Chloride (BAK) in the same manner as in the Bovine Corneal Opacity and Permeability Assay (BCOP). Control corneas were exposed to Minimal Essential Medium (MEM) only. Opacity determinations were made 10 minutes and two hours after exposure. A permeability assay was not performed. Following the opacity determinations, the corneas were removed from the holders and fixed in 10% neutral buffered formalin for histopathologic evaluation. Cytologic evaluation of the MEM in the anterior chambers of the BCOP holders were also performed. Corneas exposed to DOSS had moderate to severe sloughing of the most superficial layers of corneal epithelium. After 10 minutes of exposure, sheets of cells were partially separated from the corneas and at 2 hours after exposure the superficial layers of epithelium were largely absent. Corneas exposed to BAK had moderate to severe edema of the middle and basal layers of the epithelium. Ten minutes after exposure to BAK moderate edema of the middle layers of the cornea was noted. Two hours after exposure severe diffuse edema with vacuolation and partial separation was noted. Cytological evaluation revealed corneal epithelial cells in the chamber fluid from corneas exposed to DOSS for 2 hours, but not in the other preparations.

## INTRODUCTION

Previous evaluations<sup>1</sup> of aqueous dilutions of anionic and cationic surfactants produced different responses in the Bovine Corneal Opacity and Permeability Assay (BCOP). The opacity responses noted in the corneas exposed to cationic surfactants were similar at 10 minutes and two hours after exposure, and increased with greater concentrations, and generally corresponded with increases in Draize eye scores. However, some anionic surfactant solutions produced high opacity responses at 10 minutes after exposure and very low opacity responses at two hours after exposure. High concentrations of anionic surfactants also produced permeability scores which were lower than expected based on Draize eye results.

The data from the previous evaluation of Dioctyl Sulfosuccinate (DOSS) is presented in the following table:

Table 1

BCOP <i>IN VITRO</i> and DRAIZE SCORES						
SURFACTANT	AQUEOUS DILUTION %	DAY 1 DRAIZE MTS	DRAIZE CATEGORY	BCOP <i>IN VITRO</i> SCORE <sup>a</sup>	CORRECTED MEAN OPACITY SCORE	CORRECTED MEAN OD SCORE
Dioctyl Sulfosuccinate	0.05	0	-	-0.67	-3.1	0.162
	0.10	0	-	8.29	7.6	0.046
	0.50	0	-	19.93	18.1	0.122
	1.0	0.67	-	17.09	12.8	0.286
	2.0	5.33	i	28.88	23.5	0.392
	5.0	13.67	+	12.47	10.0	0.166
	10	24.33	+	11.22	7.2	0.268

## KEY:

<sup>a</sup>BCOP *In Vitro* Scores = Corrected Mean Opacity Score + 15 (Corrected Mean Optical Density Score)

- = negative

+ = positive

i = indeterminate

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We expected the BCOP *In Vitro* Score to increase in a similar manner as the Draize Mean Total Score increases. However, the *In Vitro* Score peaked at a DOSS concentration of 2.0% and decreased at 5.0 and 10%, even though the Draize Scores increased. This was not noted with the cationic and non-ionic surfactants. Benzalkonium Chloride (BAK) was chosen for use in this study since it produced a typical response in the BCOP, i.e., as the concentration of BAK increased, the Draize Scores increased and the *In Vitro* Scores increased as indicated in the following table:

Table 2

BCOP IN VITRO and DRAIZE SCORES						
SURFACTANT	AQUEOUS DILUTION %	DAY 1 DRAIZE MTS	DRAIZE CATEGORY	BCOP IN VITRO SCORE <sup>2</sup>	CORRECTED MEAN OPACITY SCORE	CORRECTED MEAN OD SCORE
Benzalkonium Chloride	0.1	2.00	-	4.02	3.9	0.008
	0.2	9.33	+	10.86	9.6	0.084
	0.3	15.33	+	23.97	15.9	0.538
	0.5	16.00	+	36.12	28.0	0.5415

KEY:  
<sup>2</sup>BCOP *In Vitro* Scores = Corrected Mean Opacity Score + 15 (Corrected Mean Optical Density Score)  
- = negative  
+ = positive

The objective of this study was to attempt to determine the histological differences in Bovine corneas exposed to DOSS and BAK at the 10 minute and two hour evaluation periods, and to examine the Minimal Essential Medium (MEM) in the cornea holders to determine the presence of cellular components of the cornea which may have been stripped off by the action of the chemicals.

## **MATERIALS AND METHODS**

This method was based on the protocol established by Gautheron et. al.<sup>2</sup> as well as the Standard Operating Procedures provided with the OP KIT™ opacitometer.<sup>3</sup> The bovine eyes were received from Spear Products, Quakertown, PA and transported to MB Research Laboratories in Hanks Balanced Salt Solution in a refrigerated container. The eyes were examined within one hour after receipt and any eye with a cornea exhibiting evidence of vascularization, pigmentation, opacity, or scratches was discarded. Corneas from eyes which were free of defects were dissected from the surrounding tissues. A 2-3 mm rim of sclera was left attached to each cornea. The dissected corneas were mounted in specially designed holders segmented into anterior and posterior chambers which were filled separately. Each cornea was mounted to allow the corneal epithelium to project into the anterior chamber. The posterior chamber was filled with Minimal Essential Media supplemented with 1% Fetal Bovine Serum (MEM). The anterior chamber was then filled with MEM. Each cornea was visually inspected again to insure that there were no defects. The entire holder with the cornea was submerged in a 32°C water bath and allowed to equilibrate for at least one hour, but not longer than 2 hours. Following equilibration, the holders containing the corneas were removed from the water baths. The MEM was removed from the chambers and the chambers refilled with fresh MEM.

Measurements of opacity through the cornea were made using an OP-KIT™ opacitometer produced by Electro-Design Corporation of Rion, France. Each treated cornea was scored in comparison to two control corneas. The pre and post-exposure determinations of opacity for the MEM treated controls were made by measuring against the blanks supplied with the opacitometer. Following the pre-exposure determinations, the MEM was removed from the anterior chamber and a volume of 0.75 ml of the test article was applied to the epithelium of each treated cornea through the access port in the holder. After  $10 \pm 1$  minute, the test article was removed from the epithelium of the cornea and the anterior chamber by washing with MEM. All holders were then refilled with fresh MEM and a measurement of opacity was taken comparing each of the five treated corneas to the controls. At this time, the corneas designated for the 10 minute exposure were removed from the

holders and fixed in 10% neutral buffered formalin (NBF). The remaining corneas and holders were returned to the water bath and incubated at 32 °C for an additional two hours. At the end of the two hour period, a sample of the anterior chamber fluid was added to 10% NBF for cytological evaluation. The MEM was then changed again and a measurement of opacity taken. The corneas were removed from the holders and fixed in 10% NBF. No measurements of permeability were taken since it was necessary to remove the corneas for histopathologic evaluation.

For histopathologic evaluation, a 2 mm section of the central cornea was trimmed, processed, embedded on edge in paraffin, sectioned at 5  $\mu$ m, and stained with hematoxylin and eosin (H & E). Anterior chamber fluids from the two-hour sample time were subjected to centrifugation, and the pellets were resuspended in 0.1-0.2 ml phosphate-buffered saline. Cytospin preparations of the suspensions were stained with Wright's stain and examined microscopically.

## RESULTS

Corneal Opacity - The corrected mean opacity score for each group was:

GROUP	TEST MATERIAL	CORRECTED MEAN OPACITY SCORE	
		10 MINUTES	2 HOURS
1	MEM	-1.00	N/A
2	MEM	0.67	-0.3
3	DOSS 10%	18.75	N/A
4	DOSS 10%	19.85	-0.95
5	BAK 0.5%	26.5	N/A
6	BAK 0.5%	22.90	31.7

N/A = Not Available: corneas had been fixed in 10% NBF after the 10 minute score.

**Histopathology** - MEM-treated corneas were characterized by a corneal epithelium (10-12 layers) over a much thicker stroma (Figure 2). The corneal epithelium consisted of 2-3 layers of stratified squamous (flat) cells on the surface overlying 9-10 layers of cuboidal epithelial cells. The stroma consisted of a dense pattern of collagen fibers running parallel to the corneal surface. There was no appreciable difference between corneas collected at 10 minutes and 2 hours.

DOSS-treated corneas were characterized by progressive sloughing of the superficial layers of stratified squamous epithelium over the 10 minute to 2 hour time period (Figures 3 and 4). After 10 minutes exposure, sheets of squamous epithelial cells were partially separated from the corneal surface. After 2 hours exposure, the corneal surface generally lacked the stratified squamous cell layers or isolated squamous cells protruded from the surface. Isolated epithelial cells at the surface were sometimes swollen and pale (edema). All other corneal structures appeared similar to the MEM controls.

BAK-treated corneas were characterized by corneal epithelial edema beginning with the middle layers at 10 minutes of exposure and progressing to the deep layers after 2 hours of exposure (Figure 5 and 6). After 2 hours of exposure, multifocal clefts had formed between the basal epithelium and stroma. All other corneal structures appeared similar to the MEM controls.

**Cytology** - Microscopic examination revealed corneal epithelial cells in the anterior chamber fluid from corneas exposed to DOSS for 2 hours, but not in any of the other preparations. Epithelial cells, single and in small sheets, were intermixed with bacteria and amorphous debris (Figure 7).

## DISCUSSION

The loss of the stratified squamous epithelial layers of the bovine corneas following the 10 minute exposure to DOSS correlated well with the significantly decreased opacity scores noted 2 hours after exposure (19.85 at 10 minutes vs. -0.95 at 2 hrs.). While this appears to be the primary mechanism of injury for DOSS, it was surprising that there was little increase in the permeability scores. Gautheron<sup>1</sup> noted similar responses in corneas with ionic surfactants and alcohols and suggested that materials which cause large increases in permeability be considered irritating in the absence of elevated opacity scores. Since we did not find greatly increased permeability scores with the DOSS, relying on the permeability score alone could be misleading with this class of materials. Examination of the MEM in the anterior chamber for cellular debris is a possible means of determining effects to the cornea which may not be evident using the opacity permeability scores.

Previous evaluations<sup>1,4</sup> have revealed that the *in vitro* score which approximates the boundary between irritant and non-irritant appears to be in the 4 to 10 unit range. Since the anionic surfactant concentrations which produced epithelial sloughing also produced high 10 minute opacity scores, i.e., in the 20 - 30 unit range, they would still be considered irritants.

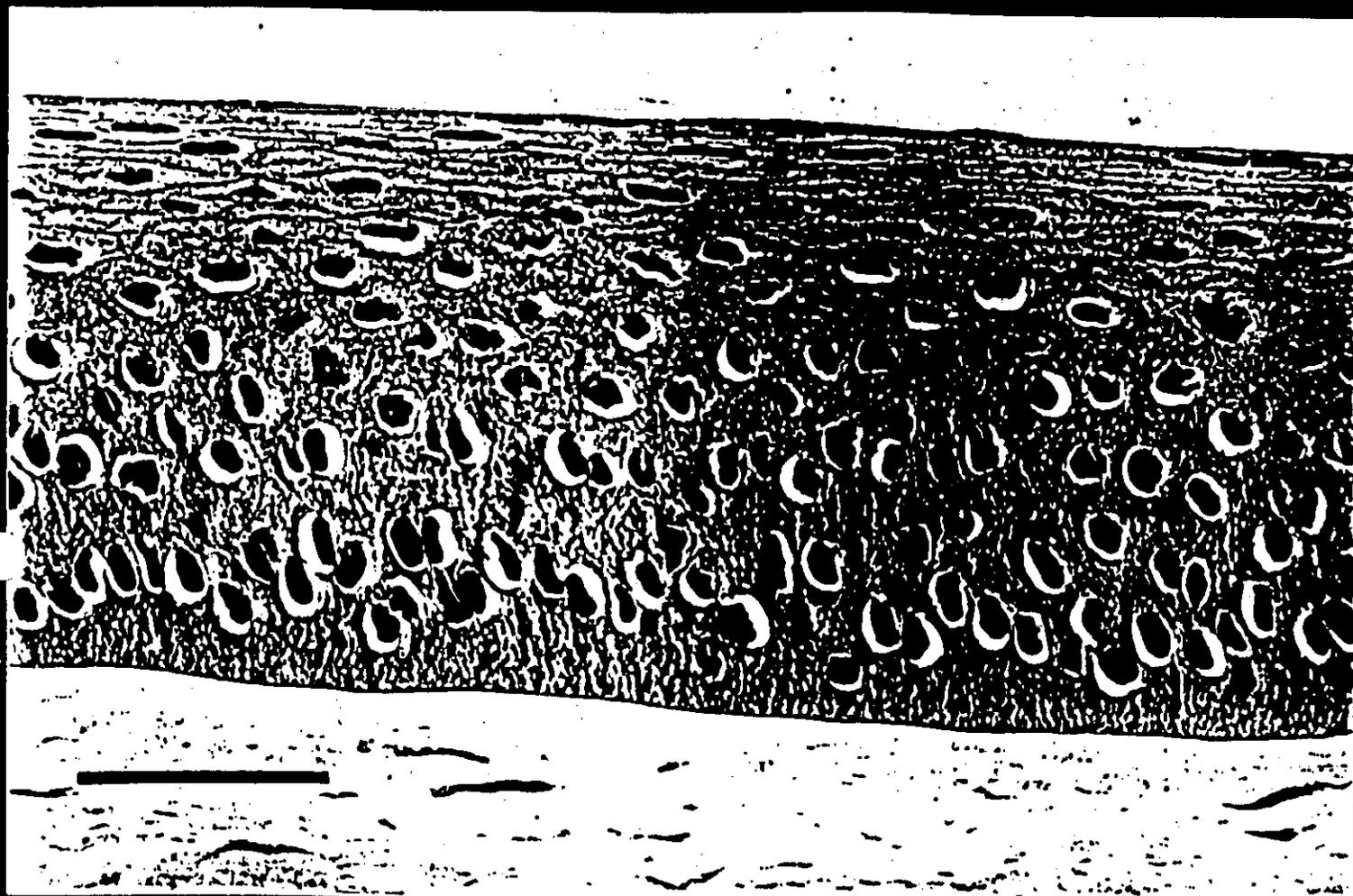
However, there is no reason to assume that the loss of corneal epithelium will always take place after the 10 minute evaluation, and it is highly possible that materials which produce false negative results in this assay may be ones which cause loss of corneal epithelium during the 10 minute exposure.

## REFERENCES

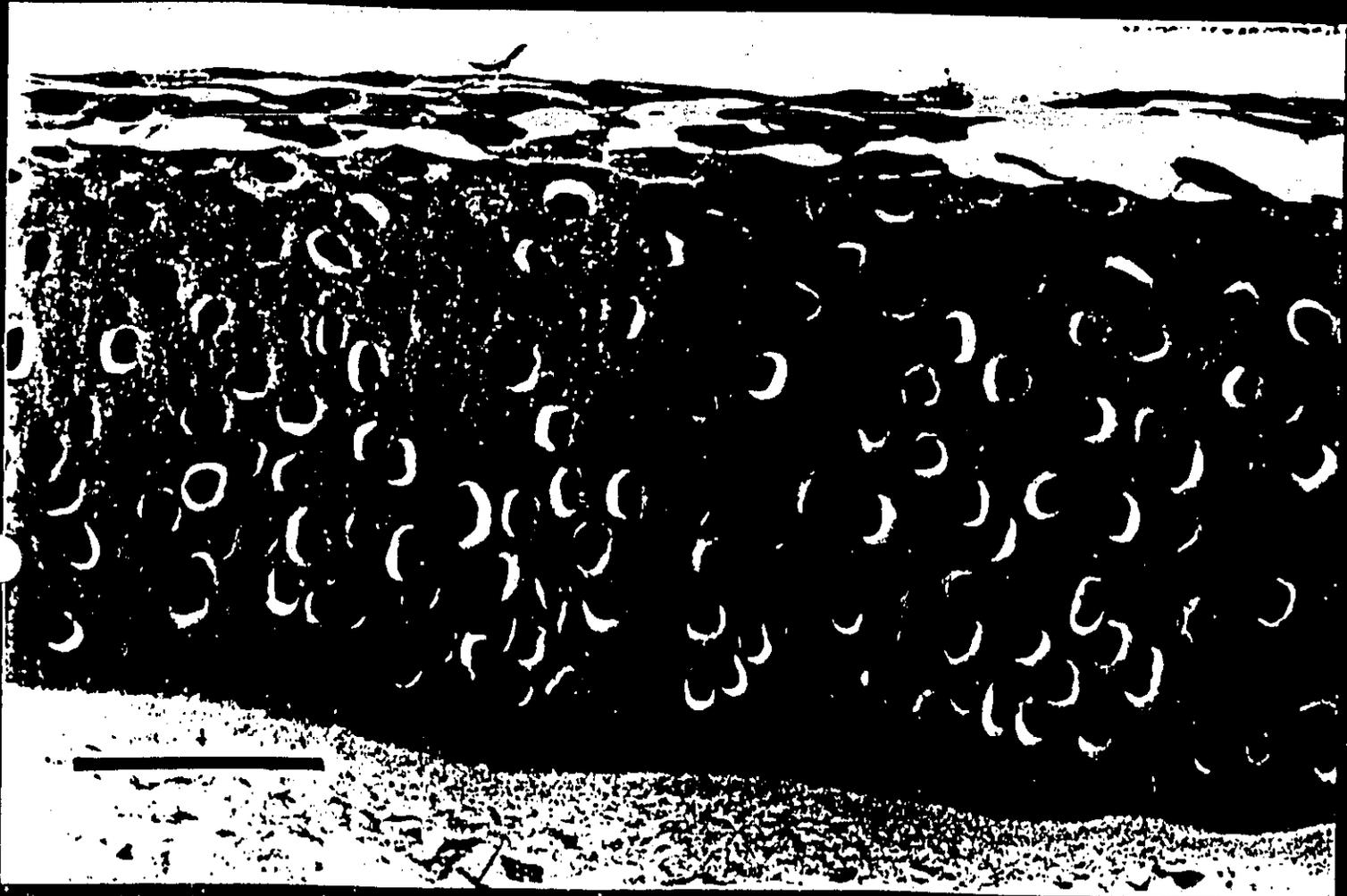
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## FIGURES

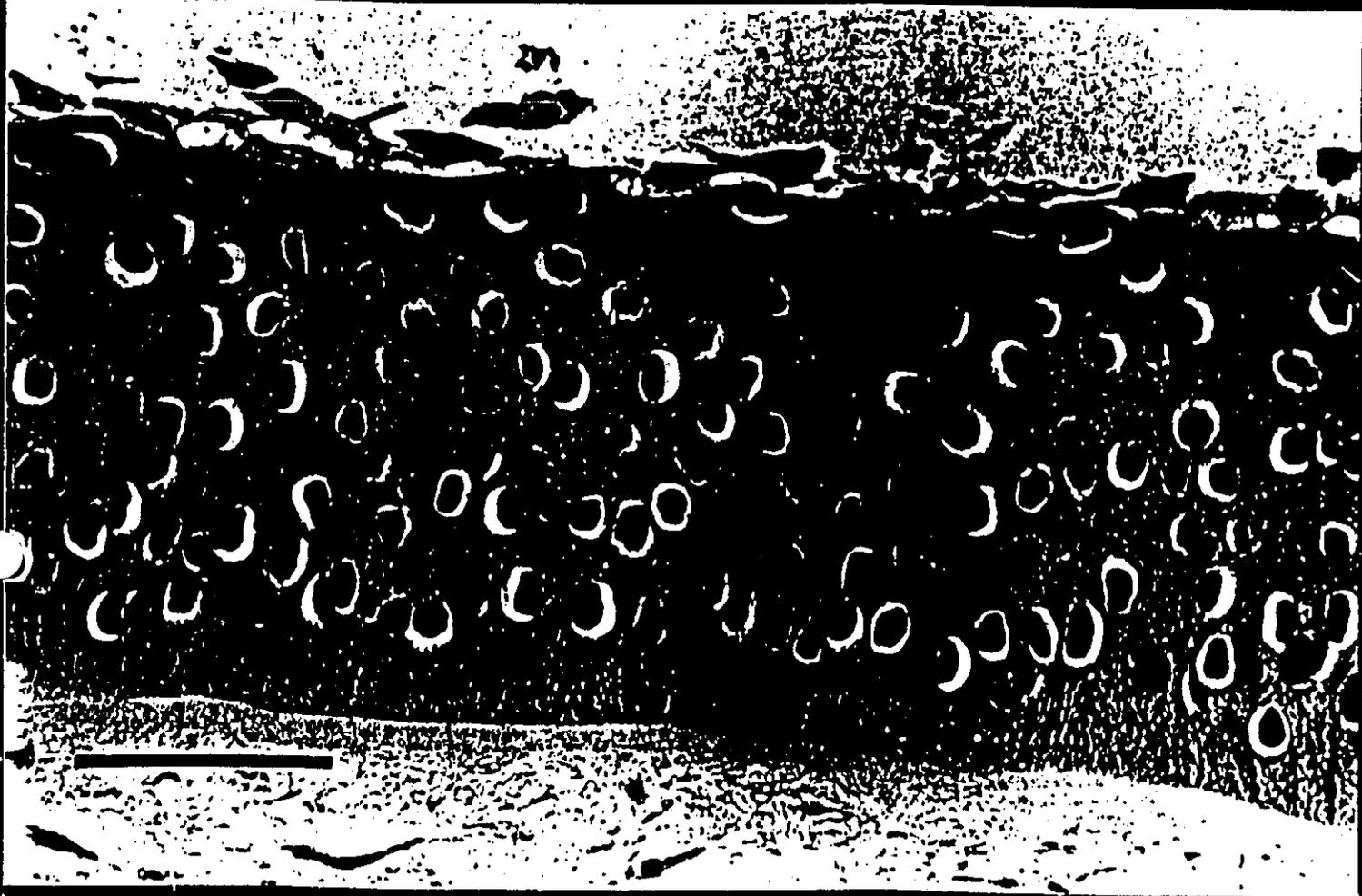
- Figure 1. Corneal Holder (This picture did not Xerox well and is not included.)
- Figure 2. Normal bovine corneal epithelium. Exposure to MEM for 2 hours. Bar = 50  $\mu\text{m}$
- Figure 3. Early separation and sloughing of superficial corneal epithelium. Exposure to DOSS for 10 minutes. Bar = 50  $\mu\text{m}$ .
- Figure 4. Loss of superficial layers of stratified squamous corneal epithelium leaving layers of cuboidal epithelium. Exposure to DOSS for 2 hours. Bar = 50  $\mu\text{m}$ .
- Figure 5. Moderate edema (swelling) of middle layers of corneal epithelium. Exposure to BAK for 10 minutes. Bar = 50  $\mu\text{m}$ .
- Figure 6. Severe edema of middle and deep layers of epithelium with partial separation at the epithelial-stromal junction. Exposure to BAK for 2 hours. Bar = 50  $\mu\text{m}$ .
- Figure 7. Small sheet of corneal epithelial cells harvested from the chamber fluid. Exposure to DOSS for 2 hours. Bar = 50  $\mu\text{m}$ . (This picture did not Xerox well and is not included.)



**Figure 2. Normal bovine corneal epithelium. Exposure to MEM for 2 hours.  
Bar = 50 $\mu$ m.**



**Figure 3. Early separation and sloughing of superficial corneal epithelium. Exposure to DOSS for 10 minutes. Bar = 50  $\mu$ m.**



**Figure 4.** Loss of superficial layers of stratified squamous corneal epithelium leaving layers of cuboidal epithelium. Exposure to DOSS for 2 hours. Bar = 50  $\mu$ m.

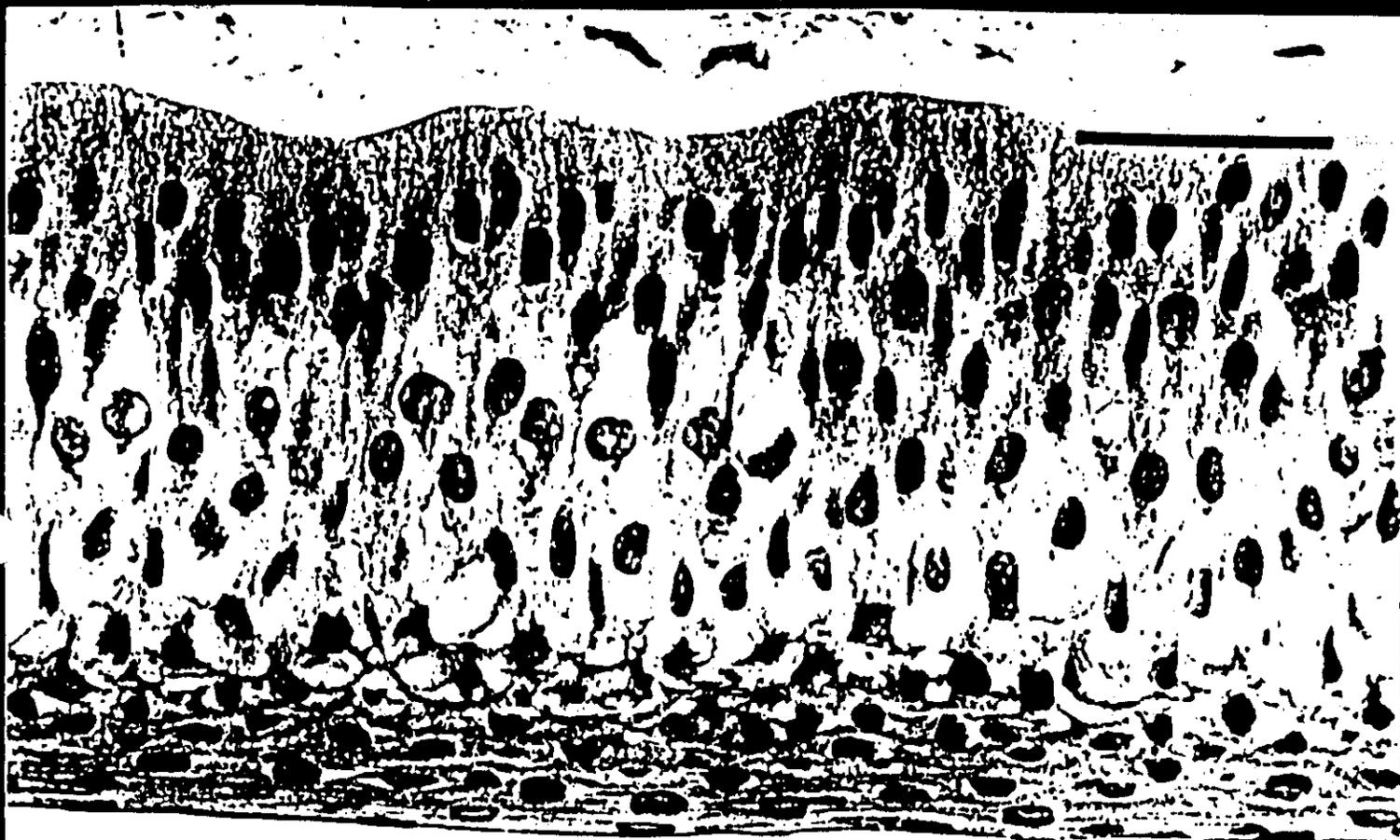


Figure 5. Moderate edema (swelling) of middle layers of corneal epithelium. Exposure to BAK for 10 minutes. Bar = 50  $\mu$ m.

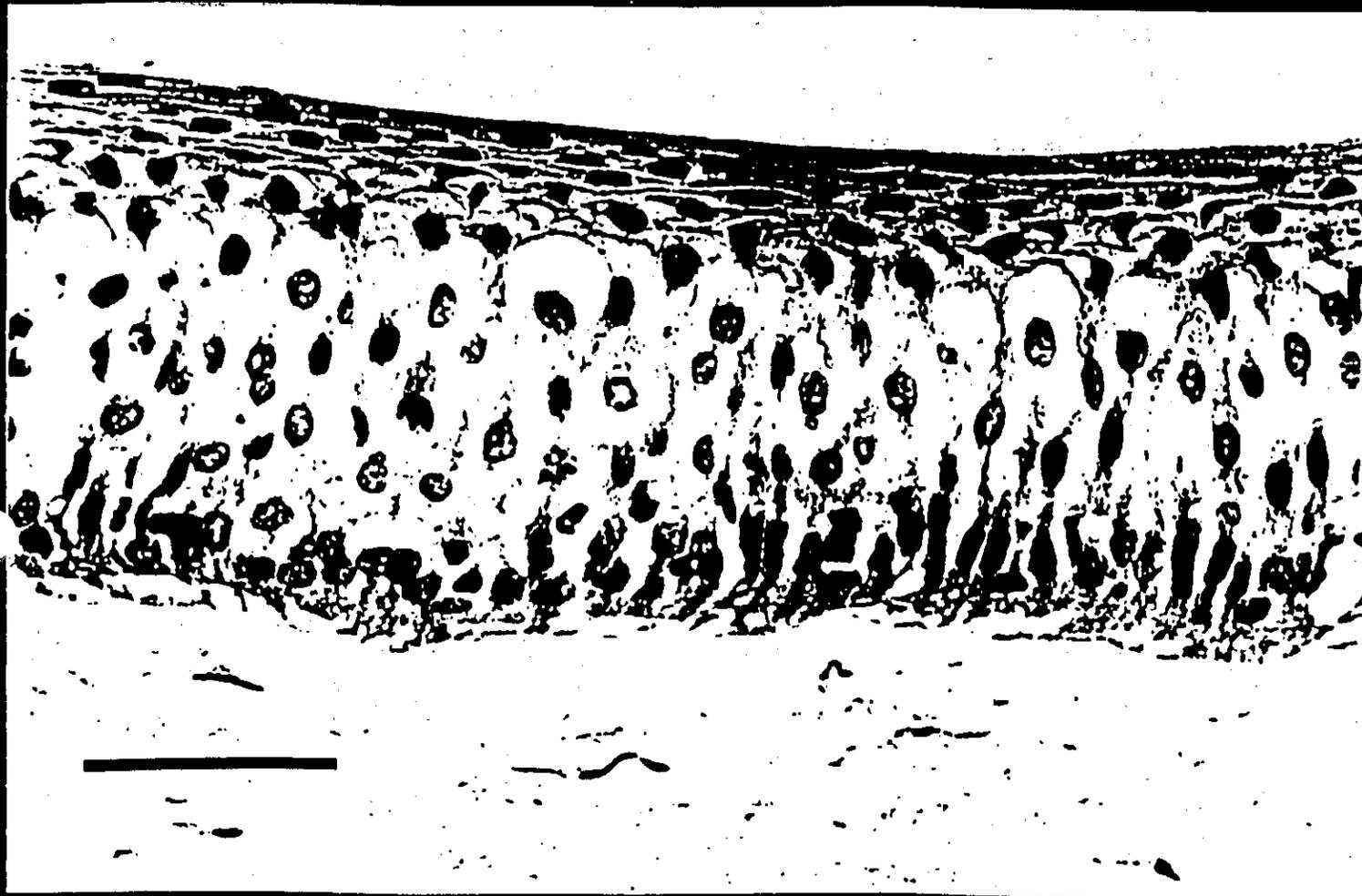


Figure 6. Severe edema of middle and deep layers of epithelium with partial separation at the epithelial-stromal junction. Exposure to BAK for 2 hours. Bar = 50  $\mu$ m.