Experimental band keratopathy electron microprobe x-ray analysis of aqueous and corneal calcium concentrations

Donald J. Doughman, Mary Jo Ingram, and William M. Bourne

Quantitative electron microprobe x-ray analyzer (EMX) assays of calcium concentrations in the aqueous and cornea of rabbits under various conditions, including experimental band keratopathy, are presented. Aqueous calcium values were significantly lowered in band keratopathy. The corneal calcium concentration remained constant in all the conditions tested throughout all layers of the cornea, with the exception of band keratopathy where an abrupt 25- to 100-fold increase occurred in the subepithelial layers. These findings are discussed, as well as our evaluation of the EMX in ophthalmology research.

Key Words: Electron probe microanalysis, band keratopathy, calcium, aqueous humor, cornea, allergic uveitis, vitamin D intoxication, rabbits, immunogenic uveitis.

We have previously reported establishing a model for band keratopathy in the rabbit. This lesion will readily develop in a rabbit's eye if, after uveitis has been induced in that eye, the animal is intoxicated with vitamin D. The pathogenesis is pleuricausal in that neither uveitis nor vitamin D intoxication alone results in band keratopathy. We presume that evaporation may be involved, since tarsorraphy prevents its development. Hypercalcemia is not seen which indicates that metastatic calcification is not the mechanism of tissue calcification in this model.

As part of our continuing study of this model, we have measured calcium concentrations in the aqueous and cornea under various experimental conditions utilizing the electron microprobe x-ray analyzer (EMX) (Fig. 1). Previous reports of EMX analysis of ocular tissue have been limited to quantitative measurements of copper and calcium in the cornea. The purpose of this paper is to report quantitative EMX analysis of the aqueous and corneal calcium concentrations in various experimental conditions, including band keratopathy.
Materials and methods

Pigmented rabbits of either sex weighing 2.0 to 3.0 kilograms were placed on a diet low in vitamin D,* housed in individual cages in a windowless room illuminated by incandescent light, and handled with gloves (to prevent endogenous production or exogenous absorption of vitamin D).

For the aqueous studies, both eyes of 10 rabbits (Group 1) were assayed for normal calcium concentrations. Twenty-four hours later, 1 mg. of egg albumin (Ovalbumin) was injected into the vitreous body in all the right eyes, inducing immunogenic uveitis in all the right eyes in approximately one week. The uveitis was observed until it was well established (approximately 3 days), then aqueous from both eyes was again sampled. At this time, systemic vitamin D intoxication was induced by intramuscular injection of 300,000 U. of vitamin D_2 (Calciferol) every other day times 3 (900,000 U. vitamin D_2 total), as previously described. Band keratopathy was well established in the right eye 24 to 72 hours after the last injection of vitamin D. The aqueous was again sampled 27 three-eighths inch disposable needle on a The entire time course for Group 1 was 18 days. In Group 2, both eyes of 5 rabbits were serially assayed at 6, 12, and 24 hours following systemic vitamin D intoxication (as described above) alone.

Aqueous (25 ml.) was obtained by transcorneal paracentesis of the anterior chamber with a No. 27 three-eighths inch disposable needle on a tuberculin syringe after topical proparacaine HCl
0.5 per cent (Ophthaine) anesthesia. Aqueous samples were prepared and calcium concentrations measured with the EMX after the method of Ingram and Hogben. For the cornea studies, the following groups were established: normal, uveitis, vitamin D intoxication, vitamin D depletion, band keratopathy, and lid closure (lid closure was performed after uveitis and systemic vitamin D intoxication, but before development of band keratopathy). One to 3 representative corneas from each group were used.

The animals were anesthetized with intravenous pentobarbitol sodium; whole corneas were removed, cut into 1 mm. square pieces with a razor blade, and immediately frozen. The tissue was dried under vacuum by slowly increasing the temperature from -80° C. to room temperature in a 2 week interval, followed by vapor fixation with osmium. This method of tissue preparation minimizes ion movement. Preparation of tissue standards and measurement of tissue calcium concentrations by the EMX were done as previously described for other elements. The experimental conditions utilized in both the aqueous and corneal studies were established by methods previously described.

In this study, an Applied Research Laboratories Electron Microprobe X-Ray Analyzer, Model 21109-59, was used. The electron beam was operated at 10 kv. with 50 namp. sample current.

### Results

Table I shows Group 1 aqueous calcium values obtained in the three conditions serially assayed: normal, uveitis, and band keratopathy. With uveitis, neither eye differs significantly from its normal condition or from its fellow eye. When band keratopathy developed, the lowering of the calcium concentration in both eyes was significant when compared with normal and uveitis, but not significantly different from each other.

In Group 2, calcium concentrations at 6, 12, and 24 hours after systemic vitamin D intoxication did not differ from the normal pretreatment condition.

Fig. 2 and 3 contain representative x-ray micrographs of the corneas probed by the EMX with the calcium values found at the various levels. The corneal calcium concentrations are similar in normal eyes, eyes with uveitis, after systemic vitamin D intoxication, vitamin D deficiency, and lid closure (Fig. 2). Also, the calcium values are similar at various levels within the cornea, including deep stroma, Descemet's membrane, and endothelium, which are not shown. In band keratopathy (Fig. 3), the epithelium and superficial stroma (as well as the deeper layers not shown) have calcium concentrations similar to normal. However, there is an abrupt, step-like, 25- to 100-fold increase in the calcium concentration in the subepithelial layer.

### Comment

Previous problems with EMX tissue analysis have been preparation of the tissue and standards. The technique of tissue and standard fixation and preparation used in this study provides reliable standards and has corneal tissue in a more physiologic state. The EMX has a spatial resolution of 1 μ allowing accurate tissue localization of the element which can be seen and photographed. For these reasons, we feel the methods used in this experiment are valid and the data obtained reliable.

The ability of the EMX to assay biological fluids has been previously demonstrated. The normal mean aqueous calcium value of 1.75 mM. per liter obtained by EMX analysis favorably compares with 1.69 mM. per liter reported by Nakamura, who used pooled samples and measured with flame photometry.

Under physiologic conditions, the calcium present in the aqueous represents diffusable calcium, i.e., calcium not bound to protein. In our normal group, slit lamp examination after each paracentesis revealed 1+ flare that had disappeared by...
### Table

<table>
<thead>
<tr>
<th>Experimental Condition</th>
<th>Value mM/L Ca&lt;sup&gt;++&lt;/sup&gt;</th>
<th>Probe Picture</th>
<th>Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>4.2</td>
<td></td>
<td>Epithelium</td>
</tr>
<tr>
<td></td>
<td>4.9</td>
<td></td>
<td>Subepithelium</td>
</tr>
<tr>
<td></td>
<td>4.5</td>
<td></td>
<td>Stroma</td>
</tr>
<tr>
<td>Lid Closure (Uveitis + Vit. D intoxication)</td>
<td>4.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uveitis</td>
<td>5.0</td>
<td></td>
<td></td>
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<tr>
<td>Vit. D Intoxication</td>
<td>4.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vit. D Depletion</td>
<td>5.6</td>
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**Fig. 2.** Representative x-ray micrograph of EMX analysis in 7 corneas under various conditions and the layers of the cornea identified. Three normal corneas and 1 cornea of the other 4 conditions were assayed. Since there was no significant difference between the layers, the values given are the mean of the entire corneal thickness. (Descemet's membrane and endothelium not shown.)

<table>
<thead>
<tr>
<th>Experimental Condition</th>
<th>Conc. of Ca&lt;sup&gt;++&lt;/sup&gt; mM/L</th>
<th>Probe Picture</th>
<th>Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bond Keratopathy (2 corneas)</td>
<td>4.2</td>
<td></td>
<td>Epithelium</td>
</tr>
<tr>
<td></td>
<td>4.4</td>
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<td></td>
<td>100-400</td>
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<td>Stroma</td>
</tr>
<tr>
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</tr>
<tr>
<td></td>
<td>4.4</td>
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</table>

**Fig. 3.** Representative x-ray micrograph of EMX analysis in 2 corneas with band keratopathy, the calcium concentration in each layer is given. Values for Descemet's membrane and endothelium (not shown) were similar to stroma.

2 hours. Since the shortest interval between samplings was 6 hours, no increased protein was present in the aliquots. In the uveitis group, 1+ to 3+ flare was present when the samples were obtained. Since 45 per cent of the serum calcium is bound to protein, we expected the aqueous calcium concentration to rise with uveitis, but such was not the case. This would indicate that, even if our technique of serial sampling induced iritis we did not detect in the normal group, it could be disregarded as a source of error in measuring aqueous calcium concentration.

The decrease in the aqueous calcium concentration in the eye with band keratopathy and its fellow control eye cannot be explained from our data. The only common event shared by both eyes is vitamin D intoxication. Our data for aqueous calcium at 6, 12, and 24 hours following systemic vitamin D intoxication show no effect. It may be that, had we sampled up to 72 hours (the time at which...
the band keratopathy group was sampled), this lowering of the calcium concentration would have been seen in the vitamin D intoxication group. We plan to study this in the future.

Quantitative values for calcium in the rabbit cornea have not previously been reported. With the use of chemical methods, 6.25 mM per liter have been reported in the human cornea.10

Our data showed an even distribution of calcium throughout all the layers of the cornea. This concentration was maintained in the face of vitamin D intoxication or depletion, as in the serum. Even in band keratopathy, the impairment of this mechanism occurs only in the subepithelial layer in an abrupt fashion, not gradual or sloped throughout the cornea. Just as lid closure prevented band keratopathy from developing clinically, lid closure was associated with a normal calcium concentration throughout the entire cornea in this study. Our data give no insight into the reasons for this. The area of increased calcium concentration we measured with the EMX in band keratopathy represents that area stained with histochemical techniques. Assuming that a histochemical stain is positive only when a critical concentration is present, the EMX could be used to determine that concentration.

Conclusion

The result of quantitative EMX analysis of aqueous and corneal calcium compares favorably with previous chemical methods. In addition, the nature of the data obtained readily lends itself to computer handling techniques, both for gathering and later manipulation. Its disadvantages are that the equipment is expensive to purchase and maintain, it requires a relatively large amount of space, and the technician must be highly trained and motivated. With tissue, the time involved in fixation and preparation is lengthy and only small amounts can be worked up at one time. Nonetheless, we feel that this technique has particular value in corneal element analysis and localization that could not have been gained as accurately by chemical methods.

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REFERENCES