tears. 7, 8 When any acid-base conjugate pair, however, resists a change in local pH, it is said to be buffering that solution. For single pairs, such buffering action is commonly described by a titration curve which indicates the amount of OH− required to shift the pH of a solution over a particular range. These functions are proportional to the concentration of the buffer and are generally S-shaped, being only approximately linear in the vicinity of the pK of the system, i.e., that pH at which an acid, for example, of such a pair is half dissociated.9

Being the dependent variable in this study, tear pH here appears as the ordinate parameter in these figures. Even so, the information contained is much the same as that represented in classic titration diagrams. Therefore the reciprocals of the slopes in these diagrams would generally correspond to the "buffer value" taken from classic titration curves, as an index of a solution’s buffer capacity.

In this study, an abrupt change was noted, however, in the buffering capacity of tears at about pH 10, a change reflective not of a single system S-shaped curve but rather of two or more systems dominating over adjacent pH ranges. Previous studies, too, have noted that the permeability of the corneal epithelium to 24Na appears to be unchanged by solutions within the range of pH 4 to 10 applied to its surface. Outside this range, however, the permeability rises, being particularly sharp on the alkaline side, so that by pH 11 the epithelial barrier is almost entirely destroyed.10 Between pH 10 and 11, then, the character of those cells seems to undergo a marked change, including perhaps even the initial stages of protein denaturation.

Indeed, as early as 1944, Friedenwald et al.,11 using a subjective 0 to 4 scaling system, were able to successfully estimate epithelial damage resulting from unbuffered solutions extending into the pH 11 range. The most significant result from that study, however, was their demonstration, by direct titration, of the buffering capacity of corneal protein, as it denatured, against alkali.

Although bicarbonate, ammonium, and phosphate systems, then, may well account for the mild, but measurable buffering effects up to (and to some extent even beyond) pH 10, the marked increase in buffering action at higher pH levels found here may indeed be a further extension of the observations of Friedenwald et al. for corneal protein, but due here to the denaturation and subsequent buffering effect of tear protein against alkali which may enter the eye.

The Ohio State University Human Review Committee approved this investigation (approval No. 756017).

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Key words: tears, human pH, buffering capacity, alkali

REFERENCES


Cornea, aqueous humor, and iris–ciliary body levels of pilocarpine and its metabolite pilocarpic acid were determined in mixed-breed rabbits following topical dosing with 25 μl of 1 × 10−3M pilocarpine. From the time-drug
concentration profile it is clear that extensive metabolism of pilocarpine occurs in the cornea of pigmented rabbits. This finding contrasts sharply with similar studies in albino rabbits where relatively low levels of pilocarpic acid were observed. It is estimated that the first-order metabolism rate constant in albino rabbits is approximately two orders of magnitude smaller than in pigmented animals. A significant observation from this finding is the possibility that the reported greater dose requirement for heavily pigmented individuals may not be due to drug-pigment binding alone but also to extensive corneal drug metabolism.

Sendelbeck et al. have reported that anterior segment metabolism of pilocarpine to pilocarpic acid and isopilocarpine in albino rabbits is rather modest. Indeed, the first-order metabolism rate constant in this multiple-dose study is of the order $10^{-4}$ min$^{-1}$. This finding was corroborated by Makoid and Robinson who reported a metabolism rate constant of $10^{-4}$ min$^{-1}$ on the basis of in vitro incubates. Similar studies in pigmented rabbits have apparently not been reported. We wish to report in this communication the surprising finding that corneal metabolism of pilocarpine in pigmented rabbits is at least two orders of magnitude greater than in albino rabbits.

**Materials.** Tritiated pilocarpine alkaloid (sp. act. 4.165 Ci mmol$^{-1}$), was purified by vacuum evaporation immediately prior to use. Male, mixed breed, dark-iride rabbits (Klubertanz, Edgerton, Wisc.) weighing between 1.8 and 2.4 kg were used throughout the study. Pilocarpic acid was prepared according to the procedures reported by Repta and Higuchi. A 25 $\mu$l volume of pilocarpine alkaloid in ethanol was incubated for 3 hr at room temperature with 100 $\mu$l of 3.5N NaOH. The reaction mixture was brought to neutrality with 1 N HCl, purified by vacuum evaporation, and lyophilized. Purity was established by thin-layer chromatography (TLC). Whatman linear LKD preadsorbent silica-gel TLC plates (Pierce Chemical Co., Rockford, Ill.) were employed for the TLC assay. The plate was developed in n-butanol saturated with 14.8M NH$_4$OH and allowed to air dry. Two centimeter sections were scraped off the plate, and the sections were transferred to scintillation cocktails for eventual scintillation counting. The approximate $R_f$ value of pilocarpine on these plates is 0.67, whereas the value of pilocarpic acid is 0.33. All other chemicals used were either reagent or analytical grade and were used as received.

**Methods**

Preparation of 0.01M pilocarpine solution. A 0.01M pilocarpine solution, iso-osmotic with tears, was prepared with pH 6.24 Sorensen's phosphate buffer. Either 0.25 or 0.50 mCi, depending on the counting efficiency required, of tritiated pilocarpine was added to the 0.01M pilocarpine solution immediately prior to each experiment. Addition of the radio labeled pilocarpine to the drug solution did not appreciably influence solution concentration, since the increase in concentration was 1% or less.

**Drug disposition studies.** A 25 $\mu$l volume of drug solution was instilled onto the cornea of fully awake rabbits. During instillation the lids were withdrawn from the globe of the eye but were immediately returned to their normal position after instillation. Both eyes of the test animal were used, but the dosing time was staggered so that one animal might, for example, be used for a 10 and 30 min time point. Animals were sacrificed at varying times, the corneal surface thoroughly rinsed with normal saline and blotted dry, and anterior segment samples obtained. Aqueous humor samples were aspirated from the anterior chamber followed by removal of the cornea and iris—ciliary body. Drug levels were then determined in each tissue.

Sixty microliter samples of aqueous humor were applied to the TLC plate and treated as described earlier.

Corneal samples were cut into four sections and soaked in 2 ml of chloroform for 18 hr. After centrifugation for 10 min at 7000 rpm, the supernatant was transferred to a holding vial. An additional 1 ml of chloroform was added to the residue, and the mixture was soaked for an additional 60 min. This also was centrifuged, and the supernatant was added to the initial extract. The solvent was evaporated to dryness under a stream of nitrogen, and the residue was dissolved in 100 $\mu$l of chloroform. A 60 $\mu$l volume of this solution was applied to the TLC plate and treated in a manner identical to that for aqueous humor. Stability of the radiolabel during the course of this assay was confirmed with controls.

Pilocarpine was quantitatively extracted from the cornea with the use of chloroform, whereas pilocarpic acid extraction efficiency was only 14.8% ± 0.2. However, the pilocarpic acid extraction efficiency was reproducible and remained at 14.8% ± 0.2 over the concentration range of interest. Extraction efficiency was determined with excised corneas and known quantities of pilocarpine and pilocarpic acid.

Iris—ciliary body samples were treated in a manner identical to that for the cornea. Pilocarpic acid extraction efficiency from iris—ciliary body
was 23% ± 1.0 and was highly reproducible.

For all samples, suitable corrections were made for background counts, and the data in counts per minute were converted to micrograms through the use of standards.

Results. A complete description of ocular disposition of pilocarpine in pigmented rabbits will be reported at a later time. Of concern in this report is the question of corneal metabolism of pilocarpine. Table I presents the percent of total drug, due to pilocarpic acid, at various times after instillation of pilocarpine in the cornea, aqueous humor, and iris–ciliary body. Each entry in the table for the cornea and iris–ciliary body was determined with corrections for extraction efficiency. Such corrections were unnecessary in the case of aqueous humor.

Statistical analysis of the data in Table I demonstrated no significant ($p = 0.05$) differences in the pilocarpic acid level in the cornea, aqueous humor, and iris–ciliary body at the 10, 30, and 60 min postinstillation points. However, at 120 mins, the pilocarpic acid level in the cornea was 1.2 times the level in the aqueous humor and iris–ciliary body, and this difference was significant ($p = 0.0038$, $F_{2,15} = 8.29$). The level of pilocarpic acid in the cornea, aqueous humor, and iris–ciliary body tended to increase with time to the point where 99% of the total radioactive label found in the cornea was measured as pilocarpic acid.

Discussion. Several analytical techniques were unsuccessfully attempted to resolve pilocarpine from its metabolites pilocarpic acid and isopilocarpic acid. The gas-liquid chromatography (GLC) procedure described by Dziedzic et al.\(^4\) for pilocarpine was unsuccessful in our hands. The assay involved derivatization of the compounds with heptafluorobutyric anhydride in the presence of trimethylamine and benzene, followed by GLC separation and identification. This procedure was found to be incapable of resolving pilocarpic acid and pilocarpine despite extensive variation in column packing and temperature conditions. High-pressure liquid chromatography (HPLC) appears to show promise as a technique to resolve pilocarpine from its two metabolites, and indeed Urbanyi et al.\(^5\) have reported an HPLC procedure for simultaneous determination of pilocarpine and isopilocarpine in pharmaceutical preparations. However, since pilocarpic acid represents the major metabolite, the TLC procedure described in this report was adopted.

Since the chromatographic assay employed in this study could not resolve pilocarpine from its other metabolite and stereoisomer, isopilocarpine, a comment on the contribution of this metabolite to the total metabolism picture is needed. Sendelbeck et al.\(^1\) have shown that isopilocarpine constitutes approximately 10% of the total metabolite (the remainder being pilocarpic acid) in the albino rabbit. Thus the extent of pilocarpine metabolism in pigmented animals is probably greater than that reported in this communication.

That extensive metabolism of pilocarpine occurs in the pigmented rabbit may be inferred from the data in Table I. Insufficient data points are presently available to calculate an accurate metabolism rate constant. However, assuming instantaneous absorption of pilocarpine, one can graphically estimate it to be in the neighborhood of $10^{-2}$min$^{-1}$, which is two orders of magnitude greater than the rate constant in albino rabbits, i.e., approximately $10^{-4}$min$^{-1}$.

It is not unambiguously conclusive that metabolism occurs primarily in the cornea although there is evidence that this is the site. The very high levels of pilocarpic acid in the cornea at all time points indicate the cornea as the site of metabolism. The 120 min point is especially noteworthy in this regard because almost all the drug in the cornea is in the form of pilocarpic acid. One can create an alternative scenario to corneal metabolism by assuming that metabolism occurs elsewhere and the metabolite is transferred to and stored in the cornea. This seems unlikely given the substantial level of pilocarpic acid at the 10

### Table I. Percent of total drug due to pilocarpic acid in the cornea, aqueous humor, and iris–ciliary body at selected times following instillation of a 0.01M pilocarpine solution

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Time (min.)</th>
<th>10</th>
<th>30</th>
<th>60</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cornea</td>
<td></td>
<td>34.5 ± 5.9(5)*</td>
<td>50.5 ± 6.0(4)</td>
<td>87.2 ± 3.8(5)</td>
<td>98.6 ± 0.4(7)†</td>
</tr>
<tr>
<td>Aqueous humor</td>
<td></td>
<td>47.4 ± 0.2(5)</td>
<td>52.7 ± 15.0(5)</td>
<td>75.9 ± 4.5(5)</td>
<td>81.7 ± 4.5(5)</td>
</tr>
<tr>
<td>Iris–ciliary body</td>
<td></td>
<td>44.3 ± 10.0(5)</td>
<td>32.7 ± 5.9(5)</td>
<td>81.7 ± 6.0(6)</td>
<td>78.9 ± 5.4(6)</td>
</tr>
</tbody>
</table>

*Mean values ± S.E.M. The number of eyes is in parentheses.
†Significantly different from aqueous humor and iris–ciliary body ($p = 0.0038$, $F_{2,15} = 8.29$).
min point, the difficulty of back-diffusion to the cornea from either the aqueous humor or the iris–ciliary body, and the known resistance to penetration of the cornea by water-soluble organic compounds like pilocarpic acid.

That extensive metabolism should occur in the cornea is not surprising. Anderson et al. demonstrated esterase activity in the cornea of the albino rabbit in the study of conversion of the prodrug dipivalyl epinephrine (DPE) to epinephrine. The metabolism rate constant in that study was 0.17 min⁻¹. What is surprising is that the corneal metabolism of pilocarpine should be so much greater in the pigmented than the albino rabbit. It is possible that either a specific esterase was involved in the corneal conversion of DPE to epinephrine or that a specific esterase for pilocarpine is present in pigmented but not albino rabbits. A third possibility may relate to the permeability characteristics of DPE and epinephrine as contrasted to pilocarpic acid; that is, esterases may be membrane-bound, and their accessibility is controlled by the mechanism of drug permeation. This issue will be resolved in subsequent studies.

Numerous issues need to be addressed as a result of this finding of extensive corneal metabolism of pilocarpine. Such issues include the site of metabolism in the cornea, corneal permeability mechanism for pilocarpine and its metabolites, binding of pilocarpic acid to pigments, and the total anterior segment disposition of pilocarpine and its metabolites.

One point that is worthy of note is the possibility that the increased dose of pilocarpine needed in dark-iride patients is due not exclusively to drug-pigment binding but also to increased metabolism in the deeply pigmented patient.

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Key words: pilocarpine, corneal metabolism, pigmented rabbits, pilocarpic acid, anterior segment metabolism

REFERENCES


A new rapid test of contrast sensitivity function utilizing spatial bandwidth equalization. J. S. DOBSON* AND P. A. DAVISON.

The clinical potential of tests of spatial contrast sensitivity function (CSF) has been limited until recently by the time taken to administer them. A new method for the rapid measurement of CSFs is described, utilizing the principle of spatial bandwidth equalization (SBE). The method involves electronic generation on a cathode ray tube (CRT) of a sinusoidal grating pattern of increasing spatial frequency in the horizontal meridian and decreasing contrast in the vertical meridian. The observer has separate control of 10 vertical segments of pattern containing different spatial frequency bands; he is required to adjust the rate of change of contrast of each band until each grating pattern disappears at the same vertical height on the CRT. The SBE method is far more rapid than conventional methods, taking only about 6 min, and has the advantage over other recently described methods of allowing simultaneous comparison of the apparent contrast of a wide range of spatial frequencies, thus minimizing problems of criterion shift during the test. The SBE method is shown to produce comparable results with those obtained with the conventional method and is thought to have application to a wide range of clinical conditions in which CSF tests have been reported to have advantages over visual acuity tests.

In recent years a growing interest has developed in the application of the contrast sensitivity