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The role of esterase activity in the ocular disposition of dipivalyl epinephrine in rabbits

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Summary

Dipivalyl epinephrine (DPE), a clinically useful prodrug of epinephrine in the treatment of glaucoma, relies on hydrolysis by ocular esterases for expression of its pharmacological activity. This study demonstrated that in rabbits, variations in esterase activity with age and iris pigmentation did not alter the fraction of DPE absorbed by the eye and its adnexa at 10 min post-instillation of 0.1% DPE solutions. Specifically, about 10% of the instilled dose was absorbed into the eyes of albino and pigmented rabbits, 6 and 12 weeks of age. Nonetheless, the concentration of epinephrine, derived from hydrolysis of DPE, in the aqueous humor and selected ocular tissues correlated with age- and pigmentation-related variations in esterase activity, and was between 2.5 and 5 times higher than the concentrations achieved following the topical instillation of 0.1% epinephrine solutions. The results also indicated that absorption and the subsequent hydrolysis of DPE in the conjunctiva accounted for 60–75% of the instilled DPE recovered in the eye and its adnexa.

Introduction

Dipivalyl epinephrine (DPE) is a clinically useful prodrug of epinephrine (Kass et al., 1979). It owes its therapeutic advantage over epinephrine to its greater uptake largely due to its lipophilicity (Hussain and Truelove, 1976). In albino rabbits DPE was found to be hydrolyzed rapidly to epinephrine in the corneal epithelium; the

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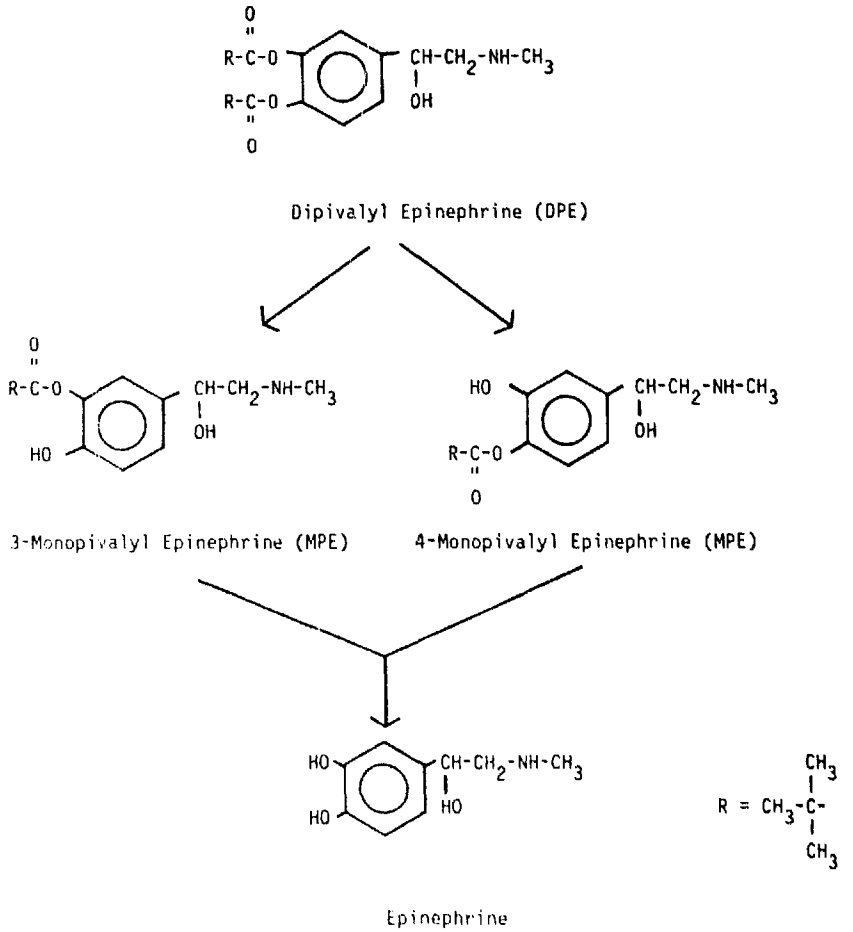
associated half-life was about 6 min (Wei et al., 1978; Anderson et al., 1980). However, the influence of variations in corneal esterase activity in altering the extent of ocular uptake and hydrolysis of DPE has not been studied. Previous studies (Lee et al., 1982; Lee and Robinson, 1982) indicated that corneal esterase activity varied with the rabbit's age and iris pigmentation, and that iris pigmentation promoted ocular drug uptake. The primary objective of this study was to investigate whether the extent of uptake as well as hydrolysis of DPE was influenced by differences in corneal esterase activity existing among albino and pigmented rabbits, 6 and 12 weeks in age.

In this report, we will interpret our data on the hydrolysis of DPE according to Scheme 1 (Wei et al., 1978; Anderson et al., 1980). We will refer to 3- and



Scheme 1. Hydrolysis of dipivalyl epinephrine.

4-monopivalyl epinephrine in this scheme collectively as monopivalyl epinephrine (MPE), simply because they migrated with the same R_f value of 0.4 in our thin-layer chromatographic system. We judged the compound with this R_f value to be MPE on the basis of two observations: (1) its biphasic concentration–time profile from the incubation of DPE with pig liver esterase; and (2) its further hydrolysis to epineph-

rine upon incubation with pig liver esterase following its recovery from the first incubation mixture.

Materials

Albino and pigmented rabbits, 6 and 12 weeks old, were purchased from ABC Rabbitry (Pomona, CA). Unlabeled and [^{14}C]-labeled (−)-dipivalyl epinephrine-HCl (1 mCi/mmol) were a gift of Allergan Pharmaceuticals (Irvine, CA). Unlabeled (−)-epinephrine-HCl was purchased from Sigma Chemicals (St. Louis, MO). [^{3}H]-labeled (−)-epinephrine-HCl (13.5 Ci/mmol) was obtained from New England Nuclear (Boston, MA), and the solvents in which it was dissolved were evaporated by vacuum distillation prior to use. Both labeled compounds were found to be over 99.5% pure radiochemically using the thin-layer chromatographic system outlined below.

Methods

(1) Preparation of dosing solutions

Solutions of DPE and epinephrine (0.1%) for in vivo studies were prepared fresh in a 0.01 N acetate buffer at pH 4 (Wei et al., 1978). Solutions (0.01% or 0.0125%) for in vitro studies were prepared fresh in an isotonic phosphate buffer at pH 7.4. Each milliliter of these solutions contained approximately 5 μCi of radioactive material.

(2) In vitro studies

(A) Hydrolysis of dipivalyl epinephrine by esterases in the homogenates of the corneal epithelium

To evaluate whether the concentration of intact DPE detected in the corneal epithelium correlated with esterase activity, 10 μl of a corneal epithelial homogenate were incubated with 40 μl of a 0.0125% DPE solution at 37°C. At 10, 30 or 60 min, the reaction was terminated by adding 10 μl of acidified methanol to the reaction mixture. Following centrifugation, 50 μl of the supernate were applied to a Whatman linear LKD preadsorbent silica gel TLC plate (Pierce Chemicals, Rockford, IL), which was then processed for chromatographic separation as described below. Incubations were performed in triplicate, and the esterase activity was calculated from the slope of a plot of dipivalyl epinephrine concentration against time normalized against protein concentration.

(B) Uptake of epinephrine and dipivalyl epinephrine by the iris-ciliary body

To gain insight into the unexpected lack of influence of pigmentation on the in vivo uptake of epinephrine and DPE by the iris and ciliary body, an in vitro

experiment was conducted wherein these tissues were incubated, in triplicate, with 2 ml of a 0.01% solution of the respective compound at 37°C. The concentration of this solution corresponded to the upper limit of epinephrine or DPE concentration detected in the aqueous humor at 10 min post-dosing. At 0, 10, 30 and 60 min, 25 μl aliquots were removed from the incubation medium and counted for radioactivity. At 60 min, the iris-ciliary body and the supernate in the incubation medium were processed for identification of DPE and its hydrolytic products using thin-layer chromatography.

The amount of epinephrine and DPE accumulated in the iris-ciliary body at each time point was calculated by difference between the amount of drug initially present and that present in the medium at sampling time. The reliability of this procedure was verified by comparing the calculated with the actual amount of drug recovered in the tissues at the end of an incubation period. In all cases these two quantities differed by no more than 10% (refer to Table 2).

(3) In vivo studies

A 25 μl volume of a 0.1% DPE solution was instilled onto the cornea of fully awake rabbits. At 10 min post-dosing, the animal was killed by an overdose of sodium phenobarbital solution administered via a peripheral ear vein. Its corneal and conjunctival surfaces were immediately rinsed with physiological saline and gently blotted dry with tissues. Approximately 120 μl of aqueous humor was aspirated from each eye. Half of this fluid was applied directly to a TLC plate for determination of DPE and its hydrolytic products. The remainder was transferred to a vial (BioVial, Beckman, Irvine, CA) containing 4 ml of pre-refrigerated scintillation cocktail (InstaGel, Packard Instruments, Downers Grove, IL) for radioactivity determination. In preliminary experiments the small amount of proteins in the aqueous humor was found not to affect the chromatographic separation efficiency.

The conjunctiva [1], cornea, and iris plus ciliary body were removed in sequence. One half of each tissue was digested in a tissue solubilizer (Soluene 350, Packard Instruments, Downers Grove, IL) for radioactivity determination. The other half was processed for extraction efficiency determination and for TLC separation of DPE from its hydrolytic products. It was cut into small fragments and allowed to soak in 2 ml of 0.01 N HCl–methanol, a solvent which was found not to adversely affect the chemical stability of the compounds under study. These tissue fragments were then homogenized for a total of 10 min in a total of 5 ml of acidified methanol in a ground glass tissue grinder placed in an ice bath. The homogenate was centrifuged at $1000 \times g$ at 4°C for 30 min. Five-hundred microliters of the supernate were counted for radioactivity for comparison with the radioactivity associated with the pellet and with the other half of the tissue. Based on these comparisons, the following extraction efficiencies were computed: 90% for the conjunctiva, 94% for the corneal stroma and intact cornea, and 97% for the iris-ciliary body. In all instances the coefficient of variation was less than 3%.

[1] Our conjunctival data were based on the whole tissue, not a specific region.

The remainder of the supernate was evaporated to dryness under a gentle stream of pre-purified nitrogen. The residue was reconstituted in 150 μl of acidified methanol. This solution was applied to a TLC plate, which was developed in a mobile phase consisting of 7 parts by volume of isobutanol, 1 part of cyclohexane, 1 part of glacial acetic acid and 1 part of water. After drying in an oven at 100°C for 3 min, 2-cm sections were scraped off the plate, and the sections were transferred to polyethylene vials (CMS, Fountain Valley, CA) containing 10 ml of a scintillation cocktail (Econofluor, New England Nuclear, Boston, MA). The samples were counted for radioactivity after 24 h of storage in the dark. Solutions of DPE and epinephrine in buffer as well as in supernates of the tissue homogenates were also spotted on TLC plates and developed as controls. The approximate R_f values of DPE, MPE and epinephrine, determined by eluting 0.5 cm sections of a TLC plate, were 0.56, 0.40 and 0.17, respectively, and were not altered by the proteins in the tissue samples.

In a series of experiments on the distribution of DPE, MPE and epinephrine between the epithelium and stroma-endothelium of the cornea, the corneal epithelium was scraped using a no. 11 scapel and the scrapings were transferred to 5 ml of acidified methanol in a holding vial, which was then sonicated in a bath sonicator (Bransonic-52, Branson Instruments, Shelton, CT) for extraction of DPE and its hydrolytic products. Care was exercised not to leave residual epithelium behind on the stroma, and at the same time not to include stromal fragments with the epithelial scrapings. The reliability of this procedure for separating the epithelium from the stroma has recently been verified (Friend et al., 1983).

Control experiments were conducted with 0.1% epinephrine-HCl as the dosing solution. Upon comparison with the chromatographic patterns of unlabeled, known metabolites [2] of epinephrine, metanephrine was the only one formed over a 10-min period. Because its R_f value (0.25) is close to that of epinephrine (0.17), what we refer to as epinephrine in this report is possibly a mixture of epinephrine and a small amount of metanephrine. However, at most 5% of the radioactivity recovered in a given tissue of the rabbit eye at 10 min post-dosing could be ascribed to this metabolite.

Results

(1) In vitro studies

The results on the hydrolytic rate of DPE upon incubation with corneal epithelial homogenates, summarized in Table 1, indicate that the esterase activity was higher in the 6- than the 12-week-old group. The esterase activity was the same in both breeds of the 12-week-old rabbits, but as previously reported (Lee et al., 1982) was higher in the albino breed of the 6-week-old rabbits. In comparison with 1-naphthyl acetate as a substrate (Lee et al., 1982), DPE was hydrolyzed about 10 times slower.

[2] These were metanephrine, dihydroxyphenylglycol, methoxyhydroxyphenylglycol, dihydroxymandelic acid and vanillylmandelic acid (Wei et al., 1978).

TABLE 1

IN VITRO HYDROLYSIS OF DIPIVALYL EPINEPHRINE BY ESTERASES IN THE CORNEAL EPITHELIUM

Rabbit	Initial hydrolytic rate [a] (nmol/min/mg protein)
6-week albino	5.13 ± 0.50 [b]
6-week pigmented	3.44 ± 0.34 [b]
12-week albino	2.51 ± 0.28 [c]
12-week pigmented	2.35 ± 0.18 [c]

[a] Obtained from the slope of a plot of dipivalyl epinephrine concentration vs time. Time points studied were 0, 10, 30 and 60 min. Figures reported were mean ± standard error of the mean for triplicate determinations. Figures with the same superscripts were compared for statistical significance.
[b] Significantly different at $P < 0.05$ by a Student's t-test.
[c] Not significantly different at $P < 0.05$ by a Student's t-test.

The uptake of epinephrine and DPE by the iris-ciliary body showed no time dependency under our experimental conditions. Uptake was already at its maximum when the medium was first sampled immediately following placement of the tissues in the incubation medium; these values are listed in Table 2. The pharmacologically active epinephrine exceeded the pharmacologically inactive DPE in the extent of uptake by a factor of 2–4. While the uptake of epinephrine was independent of the age of the rabbit, the uptake of its prodrug was twice as extensive in the 6- than 12-week-old rabbit, despite the lower tissue mass of the younger rabbit. In agreement with our in vivo results, pigmentation of the iris and ciliary body did not promote the uptake of either compound.

(2) In vivo studies

Within 10 min of instillation of 0.1% DPE solutions, only 30% or less of the radioactivity recovered in the eye was in the form of DPE, while over 50% of it was

TABLE 2

PERCENT UPTAKE OF EPINEPHRINE AND DIPIVALYL EPINEPHRINE BY THE IRIS-CILIARY BODY IN VITRO [a]

Rabbit	Dipivalyl epinephrine		Epinephrine	
	Calculated [b]	Direct measurement [c]	Calculated [b]	Direct measurement [c]
6-week albino	22.5 ± 0.84	22.5 ± 0.84	41.3 ± 1.15	36.5 ± 0.82
6-week pigmented	24.5 ± 1.21	24.5 ± 0.57	39.9 ± 0.47	38.3 ± 0.62
12-week albino	11.0 ± 0.61	10.3 ± 0.75	42.6 ± 0.42	38.6 ± 0.30
12-week pigmented	10.3 ± 0.75	9.2 ± 1.31	43.6 ± 0.22	43.1 ± 0.30

[a] Mean ± standard error of the mean for triplicate determinations. The figures reported represent results obtained from 60-min incubations of the tissues in 2 ml of 0.01% drug solutions.
[b] Calculated by difference in initial and final amounts of drug in the incubation medium.
[c] Determined by measuring the amount of drug in the tissue.

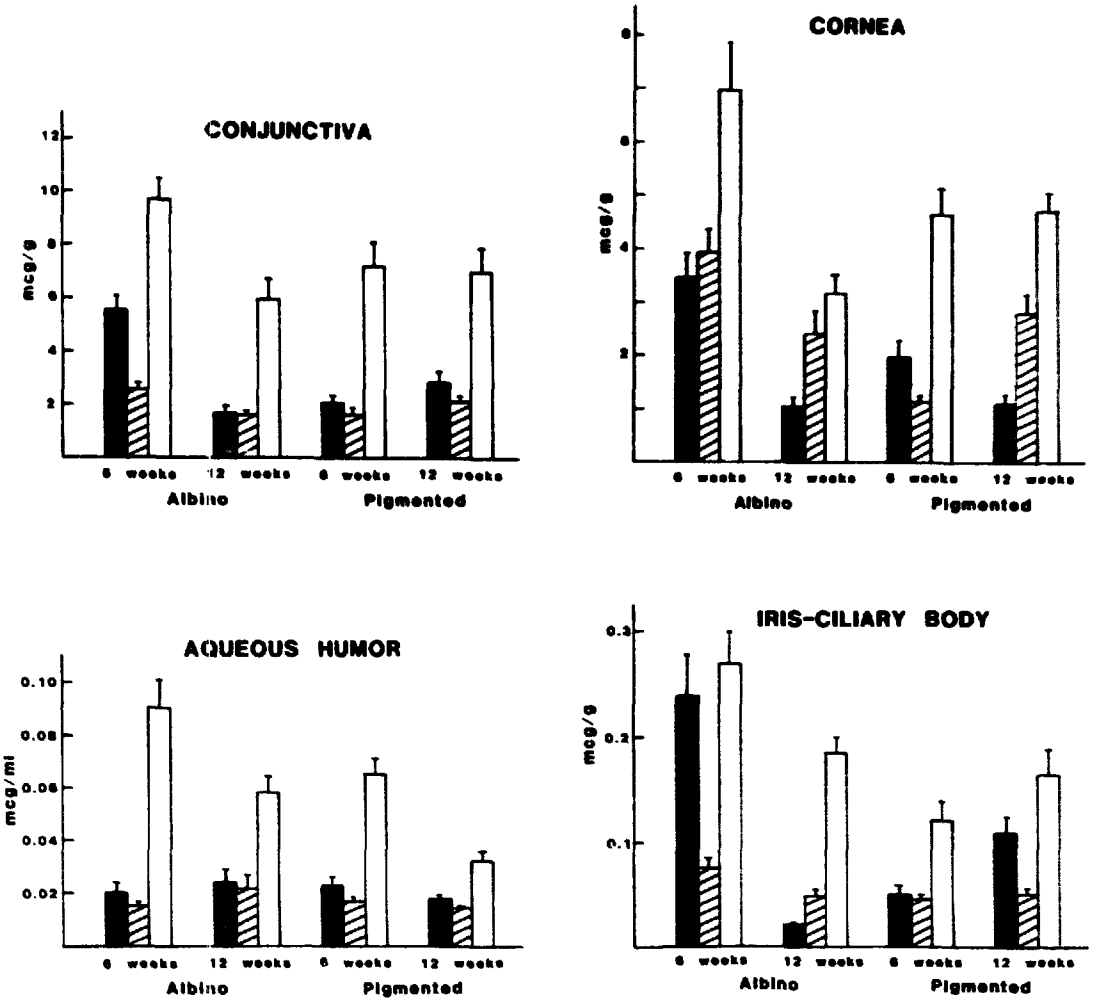


Fig. 1. Concentration (μg drug per g tissue or ml fluid) of dipivalyl epinephrine, ■; monopivalyl epinephrine, ▨; and epinephrine, □, in the conjunctiva, cornea, aqueous humor and iris-ciliary body of rabbits at 10 min following the topical instillation of 25 μl of 0.01% solutions of dipivalyl epinephrine. Approximately 18 eyes were used for each tissue or fluid. Error bars represent standard error of the mean.

in the form of epinephrine and about 20% in the form of MPE. Of these 3 entities, epinephrine consistently achieved the highest tissue or aqueous humor concentration. This is shown in Figs. 1 and 2. This trend is in general agreement with that reported by Mandell et al. (1978) and Anderson et al. (1980), who monitored the hydrolysis of DPE at later times post-dosing.

In the iris-ciliary body of the 6-week-old albino rabbit, the concentration of DPE was unusually high, and in fact was almost comparable to that of epinephrine (Fig. 1). A possible explanation is that the rate at which DPE diffused across the corneal epithelium of a 6-week-old albino rabbit was at least as fast as its rate of hydrolysis, so that a significant fraction of the DPE molecules crossing the tear/epithelium interface escaped hydrolysis.

Figs. 3 and 4 contrast the concentrations of epinephrine attained from hydrolysis

of topically applied DPE (open bars) and from topically applied epinephrine (hatched bars) in the aqueous humor and the various ocular tissues sampled. They show that in all 4 groups of rabbits studied, topical instillation of DPE was 2.5–5 times more effective than topical instillation of epinephrine in delivering epinephrine to the eye. Interestingly, despite marked differences elsewhere in the eye, the epinephrine concentration in the aqueous humor in the 6-week-old albino rabbit and

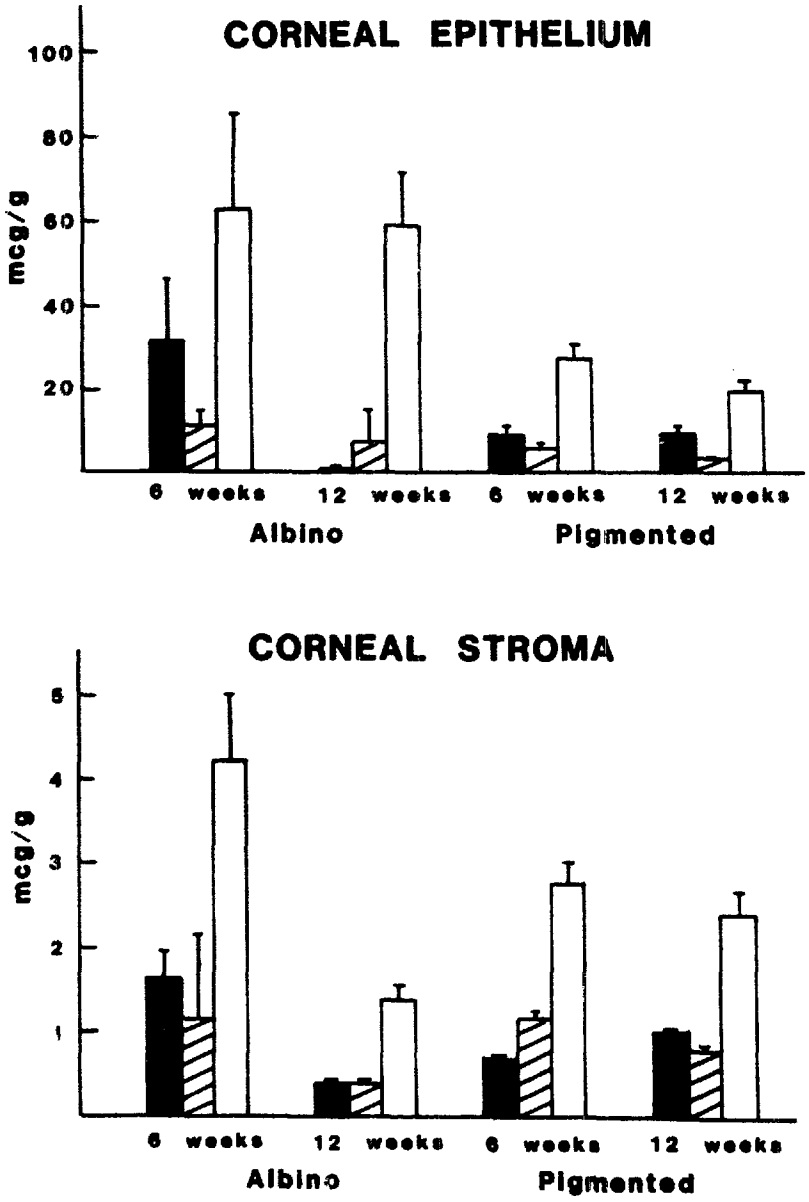


Fig. 2. Concentration (μg drug per g tissue or ml fluid) of dipivalyl epinephrine, ■; monopivalyl epinephrine, ▨; and epinephrine, □, in the epithelium and stroma-endothelium of the cornea of rabbits at 10 min following the topical instillation of 25 μl of 0.01% solutions of dipivalyl epinephrine. Approximately 18 eyes were used for each tissue or fluid. Error bars represent standard error of the mean.

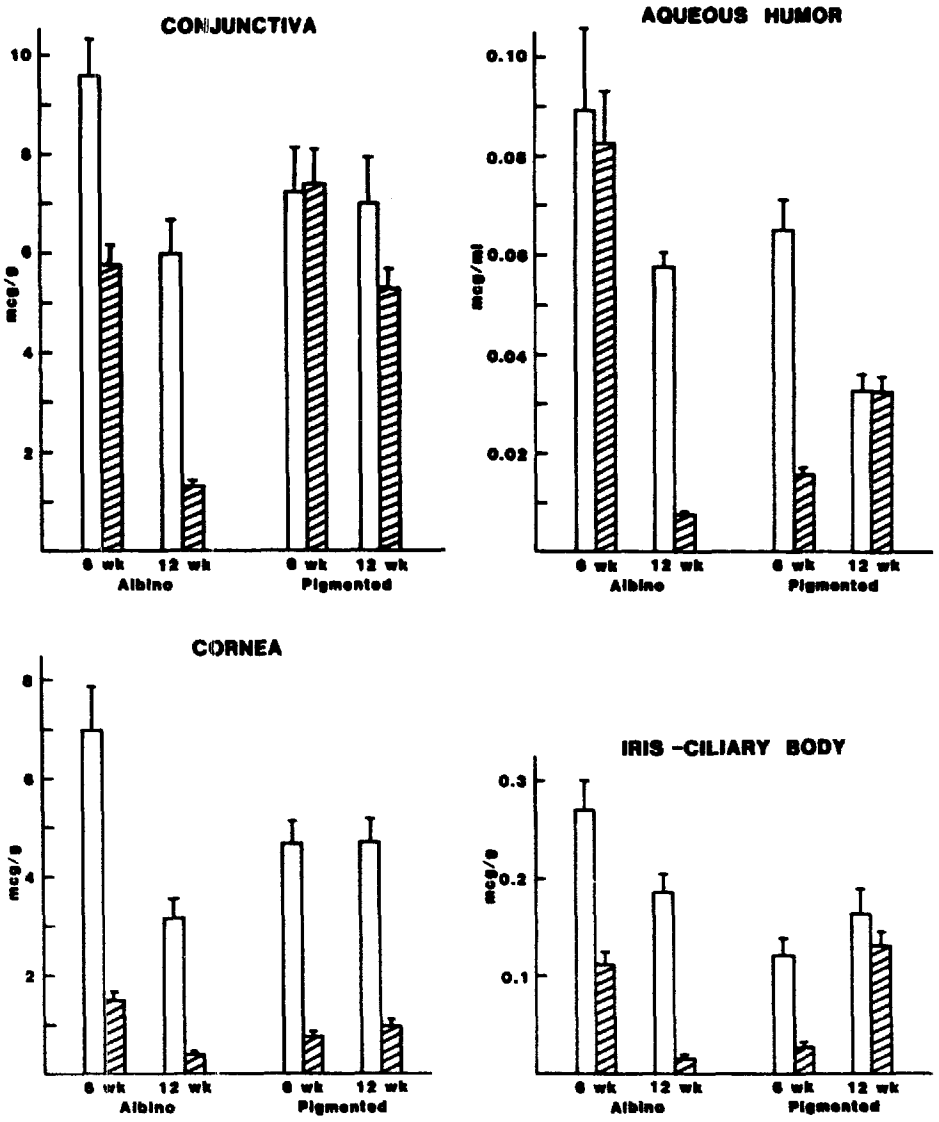


Fig. 3. Epinephrine concentration (μg drug per g tissue or ml fluid) in the conjunctiva, cornea, aqueous humor and iris-ciliary body following the topical instillation of 25 μl of 0.1% solutions of dipivalyl epinephrine, □; and epinephrine, ▨. Approximately 18 eyes were used for each tissue or fluid. Error bars represent standard error of the mean.

in the 12-week-old pigmented rabbit was equivalent from the two sources of the drug. This finding suggests that erroneous conclusions on the alteration in the ocular uptake of epinephrine would be drawn if only the aqueous humor was sampled. This conclusion has been noted elsewhere (Lee and Robinson, 1982).

Discussion

While it is generally accepted that the therapeutic efficacy of dipivalyl epineph-

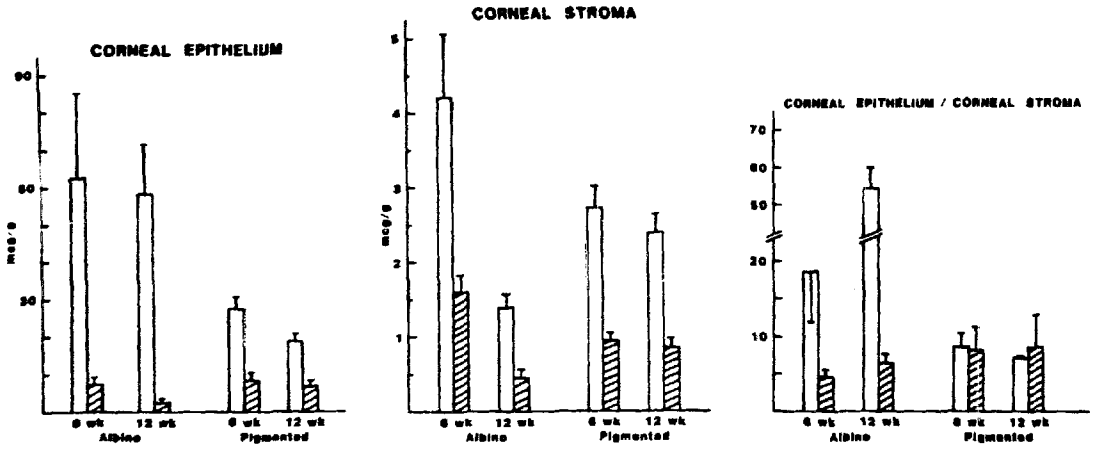
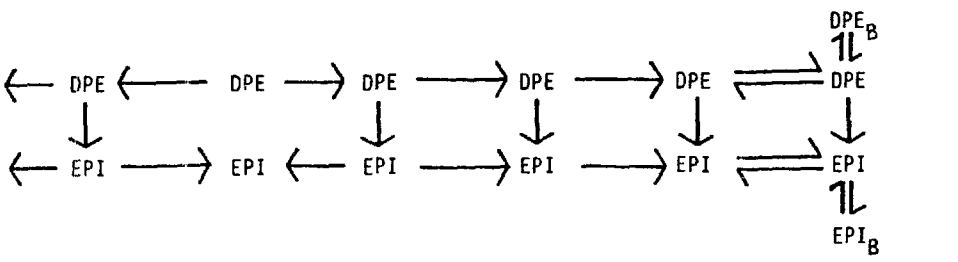


Fig. 4. Epinephrine concentration (μg drug per g tissue or ml fluid) in the corneal epithelium and stroma-endothelium, and ratio of their concentrations at 10 min following the topical instillation of 25 μl of 0.1% solutions of dipivalyl epinephrine, □; and epinephrine, ▨. Approximately 18 eyes were used for each tissue or fluid. Error bars represent standard error of the mean.

rine is linked to corneal esterase activity (Mandell et al., 1978; Anderson et al., 1980), it has yet to be determined whether its therapeutic efficacy is affected by differences in esterase activity among patients of various ages and with varying degrees of pigmentation in their irises. In the case of pilocarpine, another anti-glaucoma drug which is chemically similar to DPE in having an ester linkage as a functional group, a 3-fold increase in the least effective instilled concentration was found to be necessary for individuals with dark irises over those with light ones (Yoshida and Mishima, 1975). This was attributed to a reduction in free-drug concentration due to drug binding to the melanins in these tissues (Larrson et al., 1977). Based on their work using pigmented rabbits, Lee et al. (1980) determined that the 10-fold increase in hydrolysis of pilocarpine in the cornea of these rabbits was another factor contributing to a lower free-drug concentration in the eye.

Since both corneal drug hydrolysis and drug binding to pigments have the net effect of lowering the concentration of free, intact drug inside the eye, Lee and Robinson (1982) further postulated that both pharmacokinetic processes would indirectly promote the ocular uptake of drugs such as pilocarpine and DPE. However, we were not able to verify this hypothesis for DPE in the 4 groups of rabbits studied. The ocular uptake of DPE in these rabbits was insensitive to either the presence of iris pigmentation or variations in esterase activity with age and iris pigmentation. By summing the *amount* of DPE, MPE and epinephrine in the various ocular tissues and fluids sampled and comparing this sum to the amount of DPE applied, the percent of applied DPE absorbed into the eye and its adnexa at 10 min post-dosing averaged $9.8 \pm 1.9\%$ ($P > 0.01$ by an F-test). Conceivably pharmacokinetic processes other than the two under study may also vary with the rabbit's age and iris pigmentation, but in such a way as to diminish the impact these two processes have on the ocular uptake of DPE. Figs. 1 and 2 support this hypothesis. They show that in spite of equal ocular uptake of DPE in all 4 groups of rabbits, the

apportionment of this amount among the aqueous humor and the various ocular tissues varied with the age and the iris pigmentation of the rabbit studied. Obviously, aside from possible age- and pigmentation-related differences in the various pharmacokinetic processes depicted in Scheme 2, similar differences in ocular tissue mass



Key: DPE = unbound dipivalyl epinephrine, DPE_B = bound dipivalyl epinephrine, EPI = unbound epinephrine, EPI_B = bound epinephrine. For simplicity DPE is assumed to be hydrolyzed directly to epinephrine. Both DPE and EPI in the aqueous humor can be distributed to ocular tissues other than the iris and ciliary body. In addition, both DPE and EPI in the conjunctiva can enter the systemic circulation.

Scheme 2. Ocular distribution of topically applied dipivalyl epinephrine.

(Miller and Patton, 1981) also can account for the differences observed in the concentration of DPE and its hydrolytic products in the various ocular tissues.

Whether or not DPE would improve the ocular uptake of epinephrine ultimately depends on its rate of hydrolysis in tears relative to its transport across the tear/corneal epithelium interface. We have verified the absence of DPE hydrolysis in tears by incubating 25 μl each of rabbit tears and a 0.02% DPE solution for 10 min, which was the duration of our in vivo studies. Because of the absence of DPE hydrolysis in tears, the epinephrine found in this fluid in the in vivo study, amounting to 60–80% of the radioactivity recovered therein (data not shown), most probably derived from outward diffusion of epinephrine formed in the corneal or conjunctival epithelium. Scheme 2 considers this possibility.

While little DPE was lost to hydrolysis in tears, a substantial fraction of the instilled dose was lost to the conjunctiva. Of the applied DPE recovered in the eye and its adnexa, 60–75% of it was associated with this tissue. Furthermore, as shown in Fig. 1, a significant portion of the DPE in the conjunctiva was hydrolyzed to MPE and epinephrine, suggesting that this tissue can hydrolyze ester drugs and thus limit their access to the systemic circulation. However, the accumulation of epinephrine derived from DPE hydrolysis in the conjunctiva, especially upon chronic dosing,

may give rise to the type of external ocular toxicity symptoms reported for DPE by Theodore and Leibowitz (1979).

In spite of its loss to the conjunctiva, DPE is lipophilic enough to promote its uptake by the corneal epithelium. The lack of significant DPE hydrolysis in tears suggests that the epinephrine found in the corneal epithelium most probably derives from hydrolysis of DPE absorbed by this tissue, and that the epinephrine concentration may correlate with the indigenous esterase activity. Indeed, the middle curve in Fig. 5 shows that the epinephrine concentration found in the corneal epithelium at 10 min post-dosing generally increases with increasing esterase activity. However, in light of the fact that the DPE concentration also varies directly, rather than inversely, with corneal esterase activity (bottom curve in Fig. 5), it is likely that changes in corneal permeability is an additional factor responsible for this trend for epinephrine concentration.

For tissues and fluids that are internal to the corneal epithelium, the assumption that the concentration of DPE or its hydrolytic products is reflective of the indigenous esterase activity would be as difficult to invoke as in the case just shown for the corneal epithelium. This is because the hydrolytic products found in these tissues and fluids can be derived from diffusion of the products formed elsewhere in addition to hydrolysis of their precursors in the very tissues or fluids under study. Two such sites are the corneal stroma and aqueous humor shown in Scheme 2.

In the corneal stroma the epinephrine can be derived from diffusion of the drug formed from DPE in the corneal epithelium, as well as from hydrolysis of DPE that has diffused into this tissue. Therefore, in comparison to topical instillation of epinephrine, a smaller ratio of epinephrine concentration in the corneal epithelium relative to the corneal stroma would be expected for topical instillation of DPE. From topical dosing of epinephrine these ratios were approximately 8 in favor of the corneal epithelium (right graph in Fig. 4). Topical dosing of DPE also yielded ratios of 8 in the pigmented rabbit, but yielded ratios in excess of 8 in the albino rabbit—18 for the 6-week-old group and 54 for the 12-week-old group. Over all

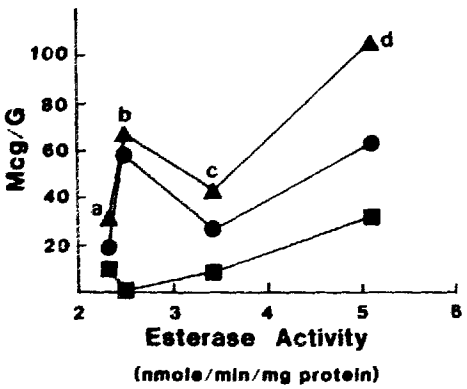


Fig. 5. Influence of corneal esterase activity on intact DPE concentration (■), epinephrine concentration (●), and the sum of concentrations of DPE, MPE and epinephrine (▲) in the corneal epithelium at 10 min following the topical instillation of 25 μl of 0.1% DPE solutions. Key: a = 12-week-old pigmented rabbit; b = 12-week-old albino rabbit; c = 6-week-old pigmented rabbit; d = 6-week-old albino rabbit.

periods of time, this accumulation of epinephrine could result in adrenochrome deposits in the cornea in susceptible individuals (Reinecke and Kuwabara, 1963).

The situation in the aqueous humor is more complex than in the corneal stroma, in that this fluid interfaces with a number of intraocular tissues to which DPE can diffuse and undergo hydrolysis and to which the hydrolytic products can return. The aqueous humor therefore can obtain its epinephrine not only from drug formed in the corneal stroma and from hydrolysis of DPE in this fluid, but also from drug formed from DPE that has diffused into the iris and ciliary body. As supporting evidence for the latter, both hydrolytic products of DPE were recovered in conjunction with DPE itself in the incubation medium following a 60-min incubation period. They amounted to 60–90% of the radioactivity in the incubation medium (data not shown).

In summary, dipivalyl epinephrine differs from pilocarpine in that its uptake into the eye and its adnexa is not significantly influenced by variations in corneal esterase activity with age and iris pigmentation, although its concentration and that of MPE and epinephrine are. While it has yet to be resolved whether differences in the regeneration rate of epinephrine from DPE would significantly influence DPE's effectiveness in lowering the intraocular pressure (Abramovsky and Mindel, 1979; Mindel et al., 1982), it seems prudent to consider adjusting the dosage regimen for DPE for changes in its hydrolytic rate caused by age- and pigmentation-related changes in esterase activity. This is simply because the intensity and duration of a pharmacological response are usually related to drug concentration.

Acknowledgements

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