Interferences—Dietary xanthines, theophylline and caffeine metabolites, and several drugs that could be administered with theophylline in a therapeutic situation, were tested for interference. The compounds that were tested and found not to interfere with the assay procedure are: acetaminophen, ampicillin, caffeine, ceforamide, cephalothin sodium, cephloridine, corticosterone, dilantin, 1,3-dimethyluric acid, 1,7-dimethylxanthine, ephedrine, hypoxanthine, isoproterenol, 1-methyluric acid, 3-methyluric acid, 1-methylxanthine, 3-methylxanthine, 7-methylxanthine, phenobarbital, theobromine, uric acid, and xanthine.

Applicability—This method has been used in the evaluation of new theophylline formulations in dogs and in bioavailability studies of theophylline formulations in healthy human volunteers. Figure 5 is a typical theophylline plasma level-time profile following the oral administration of a 500-mg theophylline dose to a human volunteer.

It has been demonstrated that theophylline can be efficiently analyzed by the automatic multidimensional HPLC technique, thus, totally eliminating the extraction step. With the use of a computing integrator (as described in this report) to automate the data reduction step, the total analysis of plasma theophylline has been reduced to manually mixing the unknown plasma sample with the internal standard solution in a small glass tube of the autosampler; the rest of the procedure is performed automatically by instruments.

REFERENCES

(1) D. H. Freeman, *Anal. Chem.*, **53**, 2 (1981).

(2) E. Erni and R. W. Feri, *J. Chromatogr.*, **149**, 561 (1978).

(3) F. W. Willmott, I. MacKenzie, and R. J. Dolphin, *J. Chromatogr.*, **167**, 31 (1978).

(4) E. J. Johnson, R. Gloor, and R. E. Majors, *ibid.*, **149**, 571 (1978).

(5) A. Yamatodani and H. Wada, *Clin. Chem.*, **27**, 1983 (1981).

(6) J. A. Apffel, T. V. Alfredson, and R. E. Majors, International Symposium on Chromatography, Cannes, France, June, 1980.

(7) J. R. Miksic and B. Hodes, *J. Pharm. Sci.*, **68**, 1200 (1979).

(8) J. J. Orcutt, P. P. Kozak, S. A. Gillman, and L. H. Cummins, *Clin. Chem.*, **23**, 599 (1977).

ACKNOWLEDGMENTS

The results were presented in part at the Pittsburg Conference and Exposition on Analytical Chemistry and Applied Spectroscopy, Atlantic City, N.J., March 1981.

The authors thank Sue Price, Toxicology Laboratories, St. Paul Ramsey Medical Center for providing patient plasma samples for cross checking theophylline methods, and Jeanne Fox for her critical review of the manuscript.

Esterase Activities in Adult Rabbit Eyes

VINCENT H. L. LEE

Received January 11, 1982, from the *School of Pharmacy, University of Southern California, Los Angeles, CA 90033.* Accepted for publication April 14, 1982.

Abstract □ The rational design of prodrugs to improve the therapeutic efficacy of existing drugs would be expedited if the nature of the *in vivo* enzymatic conditions that regenerate the drugs from their prodrugs is known. Using albino and pigmented rabbits as models, this research seeks to delineate the esterase activities in their corneas, irises, ciliary bodies, and aqueous humor, which are intimately involved in the disposition of drugs from topical dosing. This was achieved by monitoring the hydrolysis kinetics of α-naphthyl acetate, both in the presence and absence of esterase inhibitors, upon incubation with aqueous humor and homogenates of cornea, iris, and ciliary body. It was found that in both breeds of rabbits, esterase activity was the highest in the iris–ciliary body followed by the cornea and then the aqueous humor, and that multiple esterases probably existed in the aqueous humor and the ocular tissues studied. However, the esterase activity in the cornea and iris–ciliary body of the pigmented rabbit was greater when compared with the albino rabbit. Based on these results, drugs and prodrugs containing ester linkages can undergo varying extents of esterase-mediated hydrolysis while permeating the cornea and upon entering the aqueous humor, iris, and ciliary body. Moreover, in view of the differences in esterase activity that exist between the albino and pigmented rabbits, it would be necessary to employ both breeds of rabbits in evaluating the rate and extent to which ocular ester prodrugs would be converted to their parent compounds.

Keyphrases □ Esterase activities—adult rabbit eyes, disposition, hydrolysis kinetics □ Eyes, adult rabbits—esterase activities, disposition, hydrolysis kinetics □ Disposition—esterase activities in adult rabbit eyes, hydrolysis kinetics □ Kinetics, hydrolysis—esterase activities in adult rabbit eyes, disposition

Until recently, there have been few reports on drug metabolism in the eye (1). Invariably, they are concerned with polycyclic aromatic compounds entering the uveal circulation and reaching the photoreceptor cells through the choriocapillaries. Because of its dual capability to terminate the pharmacological activities of inherently active drugs and to transform inactive drugs to their active moieties, drug metabolism in the eye is an important aspect of drug action. In view of the necessarily close association between esterase activities in the various segments of the eye and the extent of metabolic alterations of drugs containing ester linkages, knowledge of esterase activities in the eye would allow a first estimation of the clinical efficacy of such drugs as pilocarpine, atropine, and dipivefrin.

During the past 5 years, the prodrug approach (2) of preparing bioreversible derivatives of existing drugs has been successfully extended to the topical ophthalmic drug epinephrine (3). In principle, several other topical ophthalmic drugs that are currently available should also benefit from this approach, since they, like epinephrine, contain functional groups amenable to ester prodrug derivatization.

For these ester prodrugs to be useful clinically, esterase activity must be available in the ocular tissues to regenerate the parent compounds. Several investigators (4–10) have evidence to support the presence of esterases predominantly in innervated ocular tissues. They include the cornea, iris–ciliary body, retina, and optic nerve. However, the level of esterase activities in each tissue and the biochemical properties of these esterases remain to be determined. It is reasonable to expect that when such information is available, the rational design of ester prodrugs would follow.

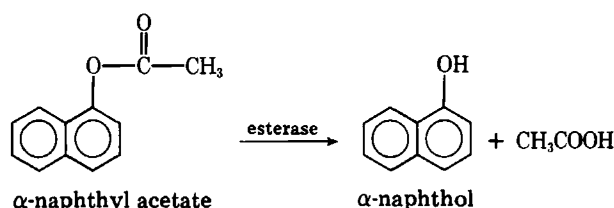
The first step in the eventual characterization of esterases present in the various ocular tissues of both albino and pigmented rabbits is the determination of esterase activities in their corneas, irises, ciliary bodies, and aqueous humor, and this is the subject of this report. The rationale for studying esterases in both albino and pigmented rabbit

0022-3549/83/0300-0239$01.00/0

© 1983, American Pharmaceutical Association

eyes comes from an earlier observation (11) that the extent and rate of hydrolysis of pilocarpine to pilocarpic acid are one to two orders of magnitude greater in the eyes of pigmented rabbits than in the eyes of albino rabbits. The cause underlying this apparent disparity is unclear, although it could be related to differences in the concentration, activities, or perhaps type of esterases.

α-Naphthyl acetate was chosen as the model substrate, since it has been shown to be a substrate for most esterases (12), and because at this stage of the study screening for esterase activity was of primary concern. The advantage offered by this substrate is that at an excitation wavelength of 317 nm and emission wavelength of 470 nm, it is practically nonfluorescent when compared with its hydrolytic product α-naphthol. Consequently, the progress of esterase-mediated hydrolysis, and therefore esterase activity, can be monitored by noting the increase in fluorescence with time. Scheme I presents the stoichiometry of the reaction.



Scheme I—Hydrolysis of α-naphthyl acetate.

Recognizing that multiple esterases are likely to be present in each ocular tissue and in the fluid and that each esterase may possess its own ester substrate specificity, the esterase activities reported here, at best, represent mean values. During preliminary investigation two other esterase substrates, ethyl p-aminobenzoate and dipivefrin, were also considered, but for a variety of reasons were found to be unsuitable to screen for esterase activity.

EXPERIMENTAL

Male albino rabbits[1], weighing ~2.4 kg, were used throughout the study. All chemicals were either reagent or analytical grade and were used as received.

Preparation of Substrate Solutions —*α-Naphthyl Acetate*—A $2 \times 10^{-3}\ M$ solution of α-naphthyl acetate[2] was prepared by first dissolving 0.0372 g of the compound in 10 ml of 95% ethyl alcohol followed by dilution to 100 ml with an isotonic tromethamine buffer at pH 7.4. From this stock solution $1.5 \times 10^{-5}\ M$ and $5.85 \times 10^{-5}\ M$ solutions were prepared for the enzymatic hydrolysis studies. These concentrations covered the range of drug concentrations typically achieved in ocular tissues.

The 10 ml of alcohol was found to be necessary to maintain the α-naphthyl acetate in solution. Two other solutions, one in 15% and the other in 20% alcohol, were also prepared to study the effect of alcohol on esterase activity. The cornea was the only tissue whose esterase activity showed statistically significant ($p < 0.05$) reduction as the ethyl alcohol concentration was varied 10–20%. Nonetheless, there was a tendency toward reduced esterase activity in the presence of ethyl alcohol. Provided a given concentration of ethyl alcohol inhibited esterase activity in the aqueous humor, cornea, iris, and ciliary body to roughly the same extent, a comparison of the relative esterase activities in them should still be valid. The remaining experiments, unless otherwise indicated, were conducted using α-naphthyl acetate solutions in 10% ethyl alcohol.

L-Leucyl-β-Naphthylamide Solutions—A solution of L-leucyl-β-naphthylamide[2], a peptidase substrate, was selected to screen for the presence of peptidases and to correct for their contribution to the esterase activity observed using α-naphthyl acetate. The peptidases, cathepsins, have been found in the eye (13) and shown to possess esterase activity (14).

A $4.67 \times 10^{-4}\ M$ solution of L-leucyl-β-naphthylamide was prepared by first dissolving 0.0114 g of the compound in 10 ml of 95% ethyl alcohol followed by dilution to 100 ml with a 0.0612 M tromethamine buffer at pH 8.2.

Fluorescence Intensities of α-Naphthol, α-Naphthyl Acetate, β-Naphthylamine and L-Leucyl-β-Naphthylamide—The wavelengths of maximum excitation and emission were determined for each compound using a spectrophotometer[3] and a spectrofluorometer[4], respectively. They were found to be as follows:

Compound	λ_{ex}, nm	λ_{em}, nm
α-naphthol	317	470
β-naphthylamine	277.5	412

The corresponding esters were found to be nonfluorescent at the wavelengths chosen. The fluorescence intensity varied linearly with α-naphthol[5] concentration in the range of 10^{-7}–$10^{-5}\ M$, and with β-naphthylamine[5] concentration in the range of 6×10^{-8}–$5 \times 10^{-5}\ M$. Potential interference of the fluorescence intensity of α-naphthol by α-naphthyl acetate was determined by spiking $1 \times 10^{-6}\ M$ solutions of α-naphthyl acetate with α-naphthol (concentrations: 0.1–$1 \times 10^{-6}\ M$). This procedure was repeated with a $1.5 \times 10^{-5}\ M$ L-leucyl-β-naphthylamide solution to which was added $1.5 \times 10^{-4}\ M$ β-naphthylamine. At these concentrations, neither α-naphthyl acetate nor L-leucyl-β-naphthylamide quenched the fluorescence due to α-naphthol and β-naphthylamine, respectively. Thus, the increase in fluorescence intensity in subsequent enzymatic hydrolysis experiments could only be due to the hydrolytic products.

Preparation of Ocular Tissues for Enzymatic Hydrolysis Studies—Albino and pigmented rabbits, approximately 10 of each, were kept in restraining boxes in a normal upright posture. They were sacrificed by a rapid injection of a 30% sodium phenobarbital solution into a marginal ear vein. After withdrawing 10 to 15 ml of blood through intracardiac puncture, the corneal surfaces were rinsed with saline and blotted dry. About 150 μl of aqueous humor was aspirated using a 27-gauge × 1.27 cm needle attached to a 1-ml tuberculin syringe, and the cornea, iris, and ciliary body[6] were removed in sequence using a surgical scalpel.

Immediately following their removal, the tissues were transferred to homogenization vessels and the aqueous humor and blood to culture tubes. All were placed in an ice bath. The tissues were homogenized in ~10 ml of ice-cold isotonic potassium chloride solution for ~80 sec using a motor-driven tissue grinder[7]. The homogenate was centrifuged at 755×g in a refrigerated (4°) centrifuge[8], and the supernatant was saved for enzymatic hydrolysis studies. The blood was allowed to coagulate, and the fibrin clot and blood cells were removed by centrifugation under the same conditions as for ocular tissue homogenates. The protein content of each supernatant was then determined using a protein–dye binding assay[9] (15) with rabbit serum albumin as the standard.

During aspiration it was possible that the aqueous humor was contaminated with esterases from the cornea and the iris–ciliary body. However, the probability of this occurring was low. This was suggested by the different response to modulators of esterase activity by aqueous humor samples as compared with cornea and iris–ciliary body samples (Fig. 1). The complicating factors, of course, were that the esterases might not partition into the aqueous humor in exact proportion to their concentrations in the tissues, and that homogenization of the tissues might release other enzymes or proteins which altered the esterases.

Enzymatic Hydrolysis of Various Substrates in the Absence of Their Inhibitors—Prior to initiating enzymatic hydrolysis studies, the presence of esterases in selected ocular tissues/fluids was ascertained by electrophoresis on 7.5% polyacrylamide gels[9] and staining proteins (16), esterases (12), and peptidases (17).

α-Naphthyl Acetate—To 3 ml of a $1.5 \times 10^{-5}\ M$ or $5.85 \times 10^{-5}\ M$ α-naphthyl acetate solution in a fluorescence cuvet was added 100 μl of ocular tissue supernatant, aqueous humor, or serum. After mixing, the

[1] ABC Rabbitry, Pomona, Calif.
[2] United States Biochemical, Cleveland, Ohio.
[3] Cary 219 spectrophotometer, Varian Instruments, Downey, Calif.
[4] Aminco-Bowman spectrofluorometer, American Instrument Co., Silver Spring, Md.
[5] Sigma Chemical Co., St. Louis, Mo.
[6] The iris and ciliary body were removed as one structure and hereafter will be referred to as the iris–ciliary body.
[7] Polytron, Brinkmann Instruments, Inc., Westburg, N.Y.
[8] Sorvall RC-5B refrigerated superspeed centrifuge, DuPont Instruments, Newtown, Conn.
[9] Bio-Rad Laboratories, Richmond, Calif.

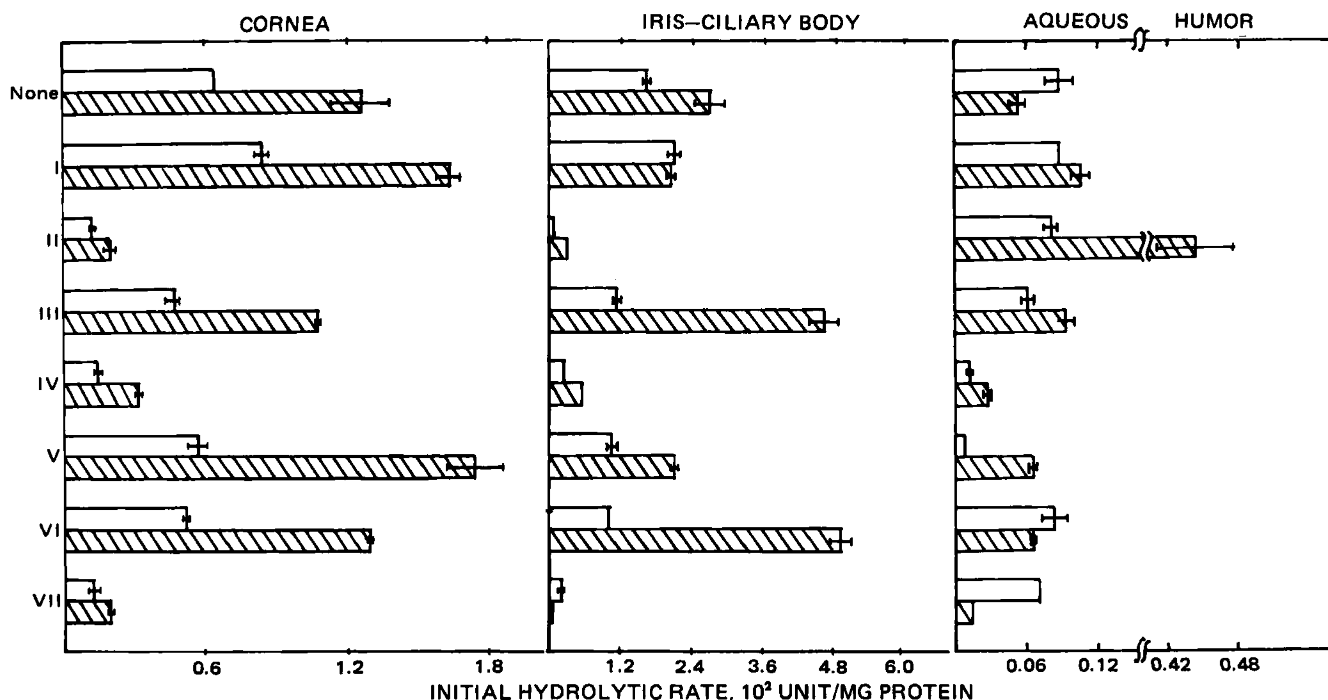


Figure 1—*Esterase activity, expressed as initial hydrolytic rate, in the cornea, iris–ciliary body, and aqueous humor of albino (□) and pigmented (▨) rabbits. Determinations were made in the presence of various inhibitors. The data for each tissue-fluid were obtained from at least 20 eyes. Error bars represent standard error of the mean. Key: I, acetazolamide; II, physostigmine sulfate; III, neostigmine bromide; IV, isofluorophate; V,* p-*chloromercuribenzoate; VI, edetic acid; VII, preheating supernatants at 45°.*

fluorescence intensity was monitored at ambient temperature every 25 sec for the first 200 sec, then every 100 sec for the next 400 sec. By applying suitable conversions, the initial hydrolysis rate in unit[10] per milligram of protein was calculated from the initial slope of a plot of fluorescence intensity *versus* time. Because no attempt was made to free membrane-bound esterases, the esterase activity thus determined should only be interpreted as that due to soluble esterases. The implicit assumption was that the fraction of soluble proteins that was esterases paralleled the total protein. As demonstrated by the lack of increase in fluorescence intensity with time in the control solution, which contained 3 ml of α-naphthyl acetate and 100 μl of 1.17% KCl, no chemical hydrolysis of α-naphthyl acetate occurred during enzymatic hydrolysis. Between three and four determinations were made for each ocular tissue or fluid of a rabbit.

The purpose of determining esterase activity in serum was to estimate the contribution of esterases in the blood, which perfuses the iris and ciliary body, to the total activity found in the iris and ciliary body. As shown in Fig. 2, this amounted to ~15% in the albino rabbit and ~25% in the pigmented rabbit.

L-Leucyl-β-Naphthylamide—One hundred microliters of tissue supernatant, aqueous humor, or serum was incubated with 2.6 ml of an activator solution[11] at 45° for 5 min. After cooling the mixture to room temperature, 300 μl of 95% ethyl alcohol was added. Hydrolysis was initiated by adding 100 μl of the amide solution to this mixture. The fluorescence intensity was monitored at ambient temperature every 100 sec for 600 sec. By applying suitable conversions, the initial hydrolysis rate in unit[10] per milligram of soluble proteins was calculated from the initial slope of a plot of fluorescence intensity *versus* time. No chemical hydrolysis of the amide occurred during enzymatic hydrolysis, as demonstrated by the lack of increase in fluorescence intensity with time in the control solution, which contained 3 ml of L-leucyl-β-naphthylamide and 100 μl of 1.17% KCl. Between three and four determinations were made for each ocular tissue/fluid of a rabbit.

The preceding procedure was also repeated with a 1.5×10^{-5} *M* α-naphthyl acetate solution to study the effect of heat on esterase activity. As shown in Fig. 1, except for the aqueous humor of the albino rabbit, the ocular esterase activity at 45° was significantly lower than that at 25°, suggesting that ocular esterases, like dermal esterases (19), were temperature sensitive.

Enzymatic Hydrolysis of α-Naphthyl Acetate in the Presence of Inhibitors—To ensure that the observed esterase activity was due to true esterases, the hydrolysis of α-naphthyl acetate was conducted in the presence of various inhibitors. They were acetazolamide[5], a carbonic anhydrase inhibitor; physostigmine sulfate[2], neostigmine bromide[2], and isofluorophate[12], all cholinesterase inhibitors; and *p*-chloromercuribenzoate[2] and edetic acid[2], modulators of carboxylesterase and aryl esterase activity. With the exception of acetazolamide, which was employed at a concentration of 2×10^{-5} *M*, the concentration of each inhibitor solution was 1×10^{-3} *M*. None of these concentrations altered the fluorescence intensity due to α-naphthol.

One hundred microliters of tissue supernatant was incubated with 2.9 ml of an inhibitor solution for 15 min prior to initiation of hydrolysis by adding 100 μl of a 4.5×10^{-5} *M* α-naphthyl acetate solution, thus generating 1.5×10^{-5} *M* α-naphthyl acetate solution. The fluorescence intensity was monitored as a function of time in the same manner as those experiments that did not involve inhibitors. This substrate concentration, 1.5×10^{-5} *M*, was well below the concentration where substrate inhibition occurred. This was demonstrated by the fact that the initial hydrolytic rate continued to increase as the α-naphthyl acetate concentration was increased to 5.85×10^{-5} *M*.

RESULTS AND DISCUSSION

The presence of esterases or esterase activity in the cornea has been implied by the hydrolysis of pilocarpine to pilocarpic acid (11) and by the conversion of the prodrugs dipivefrin (3, 8, 9) and pivenfrine (10, 20) to their corresponding parent compounds. This study demonstrated this to be the case. As shown in Fig. 3, when α-naphthyl acetate, the model substrate, was incubated with aqueous humor and the supernatant derived from the cornea, iris, and ciliary body, the fluorescence intensity increased with time, indicating that esterase activity was present in ocular tissues and fluids aside from the cornea. This esterase activity can be derived from true esterases like acetylcholinesterase, peptidases like cathepsins (14) and carbonic anhydrase (21), all of which are known or said to be present in the eye (6, 13, 22). With L-leucyl-β-naphthylamide as the substrate, peptidase activity could only be detected in the iris–ciliary body of the albino rabbit, one of the few ocular tissues enriched

[10] Unit is defined as μmoles of substrate hydrolyzed per minute.
[11] The activator solution was 0.0768 *M* in tromethamine, 1.28×10^{-4} *M* in $CoCl_2 \cdot 6H_2O$, 0.128 *M* in $MgSO_4 \cdot 7H_2O$ and 2.01×10^{-4} *M* in $MnCl_2$. It was adjusted to pH 8.2 with 3 *N* HCl. This solution was reported by Wolff and Resnick (18) to be optimal for peptidase activity.

[12] Calbiochem-Behring Corp., San Diego, Calif.

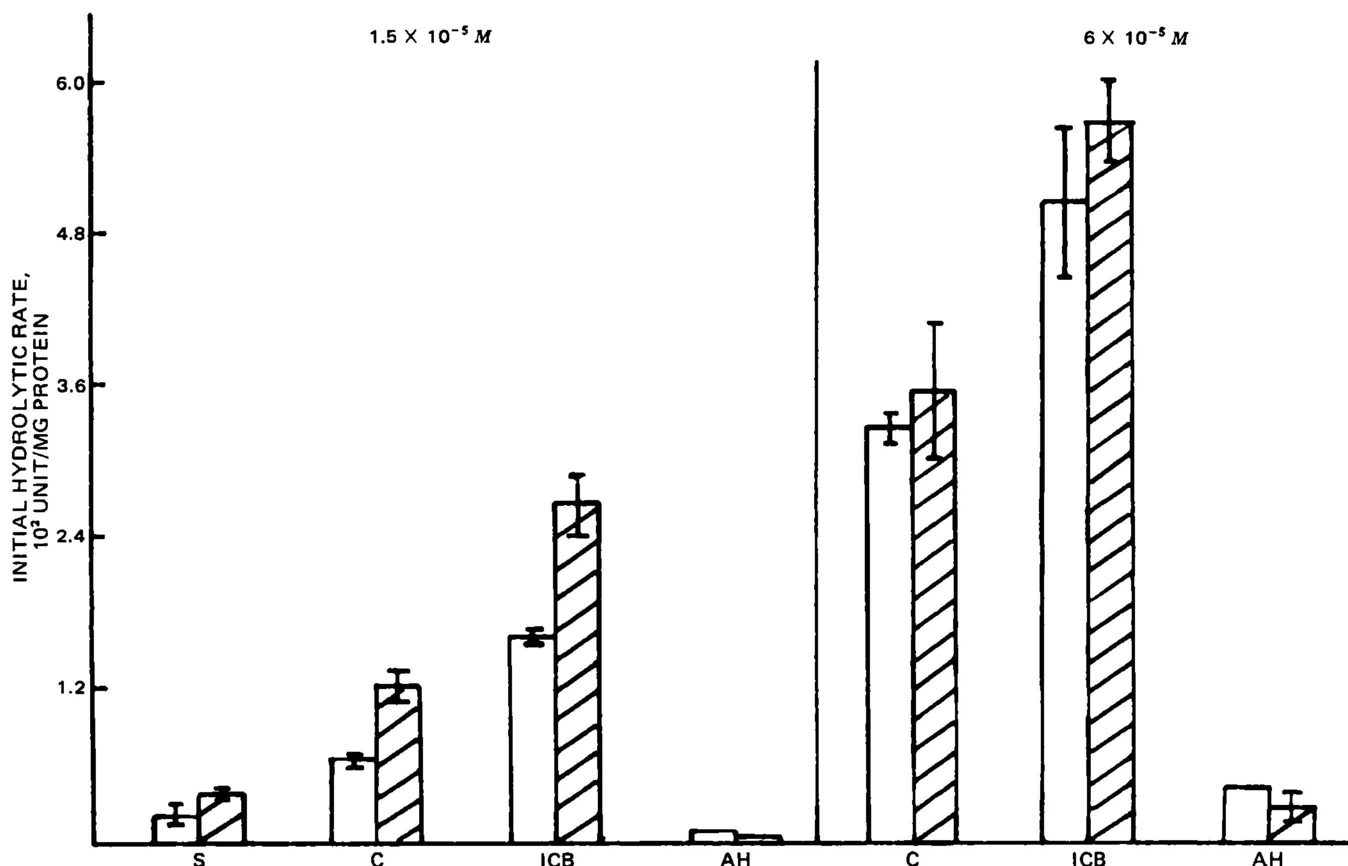


Figure 2—*Esterase activity, expressed as initial hydrolytic rate, in the serum (S), cornea (C), iris–ciliary body (ICB), and aqueous humor (AH) of albino (□) and pigmented (▨) rabbits. All determinations were made in the absence of inhibitors. The data for each tissue/fluid were obtained from at least 20 eyes. Error bars represent standard error of the mean.*

in cathepsins (13). This peptidase activity was calculated to be $3.95 \pm 0.24 \times 10^{-5}$ U/mg of protein[10], which was ~1/400 of the esterase activity in the same tissue determined under similar incubation conditions. This result, which was in agreement with previous findings (23), suggests that peptidases contributed insignificantly to total esterase activity. For unknown reasons the iris–ciliary body of the pigmented rabbit did not hydrolyze L-leucyl-β-naphthylamide and on this basis was judged to be devoid of peptidase activity.

Besides peptidases, carbonic anhydrase is another enzyme that possesses esterase activity (21), and this was verified in our laboratory[13] by incubating rabbit erythrocyte carbonic anhydrase with α-naphthyl acetate. Based on the reduction in esterase activity when acetazolamide, a specific carbonic anhydrase inhibitor that has little or no effect on true esterases (24), was included in the incubation medium, the iris–ciliary body of the pigmented rabbit was the only ocular tissue of both breeds of rabbits in which a percentage of total esterase activity could be ascribed to carbonic anhydrase. This was estimated to be 24% of total esterase activity.

Thus, true esterases were principally responsible for the esterase activity observed in the cornea, iris–ciliary body, and aqueous humor of albino and pigmented rabbits. Their behavior towards modulators of esterase activity suggested that they were a heterogeneous group of enzymes, in accord with the heterogeneity known of other esterases such as hepatic esterases (25). For the time being, two of the possible members in this group could be acetylcholinesterase and pseudocholinesterase. Their presence was suggested by the reduction in esterase activity, seen in the cornea and iris–ciliary body only (Fig. 1), in the presence of physostigmine sulfate, neostigmine bromide, and isofluorophate. Because p-chloromercuribenzoate, an agent that acts on free sulfhydryl groups in a noncompetitive fashion, also altered esterase activity in the iris–ciliary body, and also because free sulfhydryl groups have been shown to be nonessential for acetylcholinesterase activity, other esterases like arylesterase could also be present in the iris–ciliary body. As suggested by the results shown in Fig. 1, at least some of the esterases present in the aqueous humor were different from those in the cornea, iris, and ciliary body.

Taking the albino and pigmented rabbits as a group, the net effect of these esterases in the aqueous humor, cornea, iris, and ciliary body was to generate a rank order of esterase activity such that the activity was the highest in the iris–ciliary body followed by the cornea and then the aqueous humor. Specifically, the esterase activity in the cornea and aqueous humor was, respectively, 50 and 2–5% of that seen in the iris–ciliary body. The higher level of esterase activity in the iris and ciliary body may be due to the fact that these tissues are more cellular than both the cornea and the aqueous humor and, therefore, are more abundant in esterases. It may also be due to the presence of intrinsically more active esterases in the iris and ciliary body. Further work is needed to distinguish between these two possibilities as well as to identify additional ones. Nonetheless, the presence of esterase activity in the iris and ciliary body ensures that drugs containing ester linkages reaching this tissue from the systemic circulation will be hydrolyzed to some extent prior to distribution to the remaining ocular tissues.

Even though the cornea is not as enzymatically active as the iris and ciliary body, it is still in a strategic position to determine the amount of intact drug ultimately reaching the internal eye from topical dosing. The reason is that the major pathway of drug entry into the eye following topical dosing is permeation through the cornea. Partly because of this requirement, the cornea may fortuitously appear to be the primary site of hydrolysis when in fact the iris and ciliary body are enzymatically more

Table I—Michaelis-Menten Parameter Estimates for Esterases in the Cornea and Iris-Ciliary Body of Albino and Pigmented Rabbits

Tissue	K_m, $\times 10^5$ M		V_{max}, 10^2 Unit[a]/mg protein	
	Albino	Pigmented	Albino	Pigmented
Cornea	26.2	10.6	1.98	1.14
Iris–Ciliary body	15.7	3.8	1.86	0.96

[a] Unit = μmoles of substrate hydrolyzed per minute.

[13]Unpublished data.

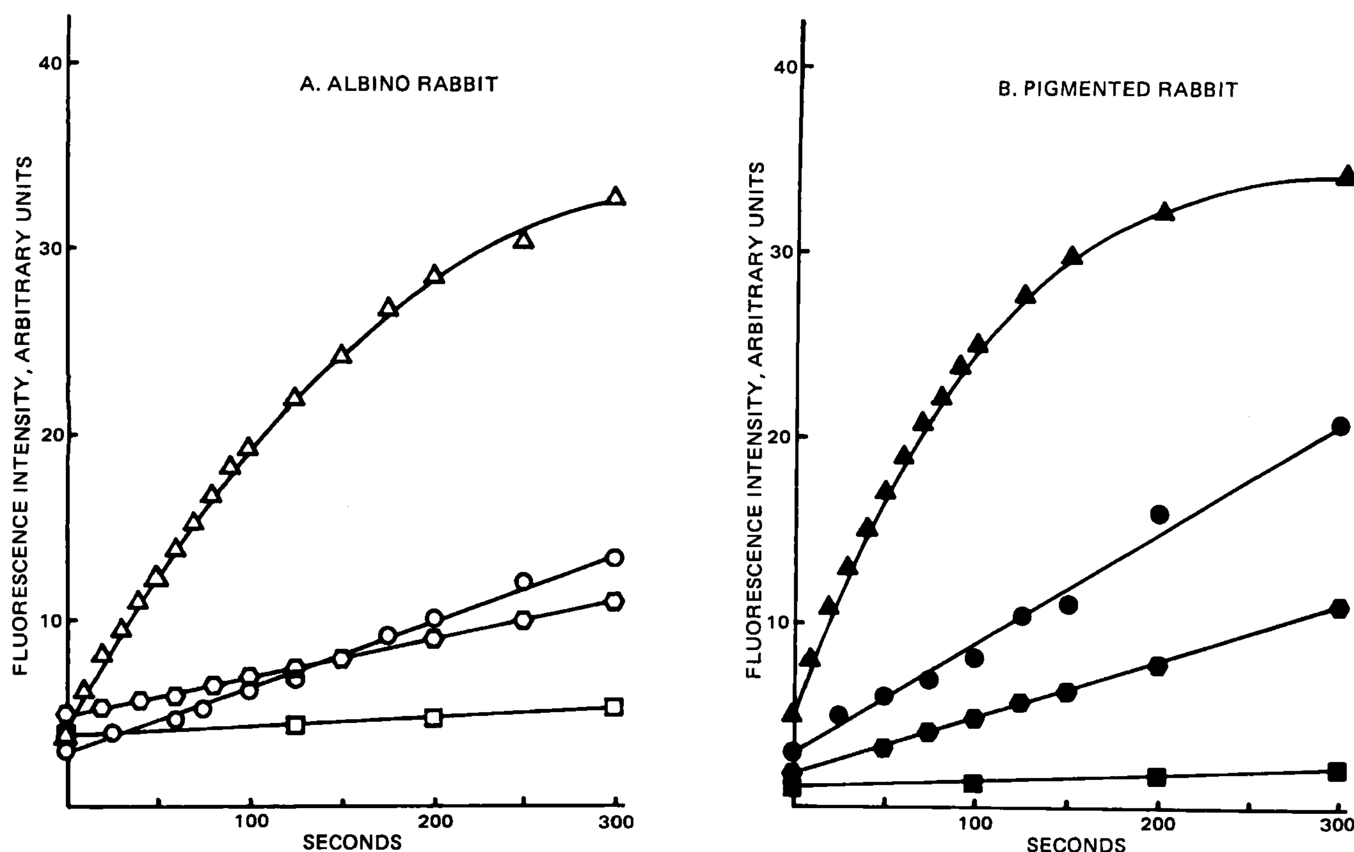


Figure 3—*Hydrolysis of α-naphthyl acetate (1.5 × 10^{-5}* M*) in the supernatants of the cornea (○, ●), iris–ciliary body (△, ▲), aqueous humor (□, ■), and serum (○, ●). The fluorescence intensity was determined at ambient temperature in a spectrofluorometer[4] with λex at 317 nm and λem at 470 nm, multiplier = 0.1 and sensitivity = 40. Plot A is shown for an albino rabbit, and B for a pigmented rabbit.*

active. This would be observed for drugs like dipivefrin (9) which are extensively hydrolyzed during permeation through the cornea.

The preceding discussion was presented in general terms, without emphasizing the possible variation of ocular esterase activity, as suggested previously (11), with rabbit strain. Based on the more extensive hydrolysis of pilocarpine to pilocarpic acid in the eye of the pigmented rabbit as compared with the albino rabbit, it was speculated (11) that the pigmented rabbit possessed greater esterase activity in the ocular tissues. This study confirmed this speculation for the cornea, iris, and ciliary body, but not for the aqueous humor. Thus, the faster rate of pilocarpine hydrolysis reported for the pigmented rabbit's cornea was more likely the result of greater esterase activity in the cornea rather than a fast permeation rate and the resultant greater accessibility of the drug to the esterases in the cornea. At an α-naphthyl acetate concentration of 1.5 × 10^{-5} *M*, the esterase activity in the pigmented rabbit's cornea and iris–ciliary body was ~2.0 and 1.6 times that in these tissues of the albino rabbit, respectively. However, such differences ceased to exist at a slightly higher substrate concentration of 5.85 × 10^{-5} *M*, suggesting that the apparent differential rate of pilocarpine hydrolysis in the two breeds of rabbits could be a substrate concentration or dose-dependent phenomenon.

Crude estimates on the Michaelis–Menten parameters associated with the ocular esterases of the rabbits, presented in Table I, suggest that the esterases in the cornea and iris–ciliary body of the pigmented rabbit show a higher affinity for the substrate than those found in the albino rabbit, but suffer from a lower maximum initial velocity. Conceivably, at a certain substrate concentration, these two factors offset one another, thereby giving identical initial rates of hydrolysis, and the esterase activity in the two breeds of rabbits would then appear to be equal. However, so long as the substrate concentration in a given ocular tissue is much less than the Michaelis–Menten constant (K_m), not at all an unlikely situation, the pigmented rabbit will appear to have a higher esterase activity than the albino rabbit.

In light of the different values obtained for the Michaelis–Menten parameters for the esterases in the cornea and iris–ciliary body of the two breeds of rabbits, it is possible that several of these esterases are unique to either the albino or the pigmented rabbit. This is further supported by the manner in which the esterase activity in the ocular tissues of the albino and pigmented rabbits responded to modulators of esterase activities, as shown in Fig. 1. Thus, in the albino rabbit, a reduction in esterase activity of the cornea and iris–ciliary body was observed with physostigmine sulfate and isofluorophate only; with the other esterase inhibitors, either an increase or no change in activity was observed. In addition, esterases which require polyvalent ions for their activity appeared to be present in the albino but absent in the pigmented rabbit.

In summary, several esterases, probably heterogeneous with respect to their substrate specificities, are present in the cornea, iris, ciliary body, and aqueous humor of albino and pigmented rabbits. In both types of rabbits, the iris and ciliary body possess twice the esterase activity detected in the less cellular cornea and 20–50 times the esterase activity detected in the acellular aqueous humor. At the concentration of α-naphthyl acetate used, the cornea and iris–ciliary body of the pigmented rabbit exhibit an esterase activity approximately double that in the same tissues of the albino rabbit. Extrapolating this observation to humans, it is probable that the greater drug requirement in brown-eye as compared to blue-eye subjects, a well-known phenomenon, can be attributed to both greater propensity of the drug for esterase-mediated hydrolysis and binding of the drug to the pigments in the iris and ciliary body.

Studies are now underway to fractionate these esterases and to characterize them with respect to substrate specificity and stereospecificity, susceptibility to substrate and product inhibition, the nature of their catalytic sites, and the pH for optimal enzymatic action. The effect of chain length on the rate of ester hydrolysis catalyzed by each esterase will also be investigated, and this information will be combined with that on the effect of chain length on tissue permeation to allow the judicial selection of a prodrug that gives the desired rate of release and even site localization. It is hoped that in so doing the bioavailability and therapeutic efficacy of drugs for such disease states as glaucoma will be improved.

REFERENCES

(1) H. Shichi and D. W. Nebert, in "Extra-hepatic Metabolism of Drugs and Other Foreign Compounds," T. E. Gram, Ed., S. P. Medical and Scientific Books, New York, N.Y., 1980, pp. 333–363.

(2) S. H. Yalkowsky and W. Morozowich, in "Drug Design," vol. 9, E. J. Ariens, Ed., Academic, New York, N.Y., 1980, pp. 121-185.
(3) P. D. Krause, in "Ophthalmic Drug Delivery Systems," J. R. Robinson, Ed., American Pharmaceutical Association, Washington, D.C., 1980, pp. 91-104.
(4) A. de Roetth, Jr., *Arch Ophthalmol.*, **43**, 1004 (1950).
(5) G. B. Koelle and J. S. Friedenwald, *Am. J. Ophthalmol.*, **33**, 253 (1950).
(6) R. A. Peterson, K. J. Lee, and A. Donn, *Arch Ophthalmol.*, **73**, 370 (1965).
(7) A. A. Swanson, *Invest. Ophthalmol.*, **13**, 466 (1974).
(8) C.-P. Wei, J. A. Anderson, and I. H. Leopold, *Invest. Ophthalmol. Vis. Sci.*, **17**, 315 (1978).
(9) J. A. Anderson, W. L. Davis, and C.-P. Wei, *ibid.* **19**, 817 (1980).
(10) W. D. Staatz, H. F. Edelhauser, R. Lehner, and D. L. Van Horn, *Arch. Ophthalmol.*, **98**, 1279 (1980).
(11) V. H.-L. Lee, H.-W. Hui, and J. R. Robinson, *Invest. Ophthalmol. Vis. Sci.*, **19**, 210 (1980).
(12) D. J. Ecobichon and W. Kalow, *Biochem. Pharmacol.*, **11**, 573 (1962).
(13) S. Hayasaka and I. Hayasaka, *Albrecht von Graefes Arch Klin. Exp. Ophthalmol.*, **206**, 163 (1978).
(14) M. L. Bender and F. T. Kezdy, *Annu. Rev. Biochem.*, **34**, 49 (1965).
(15) M. Bradford, *Anal. Biochem.*, **72**, 248 (1976).
(16) S. Fazekas de St. Groth, R. G. Webster, and A. Datyner, *Biochim. Biophys. Acta*, **71**, 377 (1963).
(17) K. Felgenhauer and G. G. Glenner, *J. Histochem. Cytochem.*, **14**, 401 (1966).
(18) J. B. Wolff and R. A. Resnick, *Biochim. Biophys. Acta*, **73**, 588 (1963).
(19) A. Pannatier, B. Testa, and J.-C. Etter, *Int. J. Pharm.*, **8**, 167 (1981).
(20) S.-S. Yuan and N. Bodor, *J. Pharm. Sci.*, **65**, 929 (1976).
(21) J. M. Armstrong, D. V. Meyers, J. A. Verpoorte, and J. T. Edsall, *J. Biol. Chem.*, **241**, 5137 (1966).
(22) G. Lönnerholm, *Acta Physiol. Scand.*, **90**, 143 (1974).
(23) J. K. Stoops, D. J. Horgan, M. T. C. Runnegar, J. de Jersey, E. C. Webb, and B. Zerner, *Biochemistry*, **8**, 2026 (1969).
(24) Y. Pocker and J. T. Stone, *ibid.*, **7**, 3021 (1968).
(25) G. Maksay, Z. Tegyey, E. Simon-Trompler, and L. Ötvös, *Eur. J. Drug Metab. Pharmacokinet.*, **5**, 193 (1980).

ACKNOWLEDGMENTS

Supported by a grant from Allergan Pharmaceuticals, Inc., Irvine, California, and by Grant EY-03816 from the National Eye Institute, Bethesda, Maryland.

Monolayer Studies of Insulin–Lipid Interactions

DAVID L. SCHWINKE, M. G. GANESAN, and N. D. WEINER ˣ

Received December 28, 1981, from the *College of Pharmacy, University of Michigan, Ann Arbor, MI 48109.* Accepted for publication April 30, 1982.

Abstract □ The interactions between insulin and various lipids were studied by monolayer penetration experiments at constant surface area. The increase in surface pressure, $\Delta\Pi$, of a lipid film depended upon the particular lipid used and the concentration of insulin in the subphase. For all lipids studied, $\Delta\Pi$ was dependent on the initial surface pressure of the lipid film. Evidence of the interaction between insulin and the lipids was found in the ability of insulin to penetrate lipid films with initial pressures >16 dynes/cm, the maximum surface pressure obtained by insulin alone. For phospholipids, both the nonpolar and polar regions influenced the degree of interaction with insulin. Saturated chain lecithins exhibited less penetration than phospholipids with unsaturated hydrocarbon chains. The net charge of the lipid was not found to be an important determinant of penetration; however, the structure of the polar group can have a dramatic effect. Insulin penetration of mixed lipid films cannot be predicted by the penetration characteristics of the pure components. The possible role of these interactions in determining the geography of the insulin molecule within the liposome and its resultant effects on the stability is discussed.

Keyphrases □ Insulin—monolayer studies of interactions with lipids □ Monolayer studies—insulin-lipid interactions □ Lipids—monolayer studies of interactions with insulin

Monolayer interactions between proteins and lipids are of much interest in the study of cell membrane structure (1, 2), hormonal action (3, 4), and enzyme activity (5). Early studies (6) established the technique of injecting a protein into the subphase beneath a lipid film at constant surface area. Since then modifications of this procedure have been used in an effort to gain a more detailed understanding of the nature of these interactions.

Recent attempts to develop an oral dosage form of insulin have utilized liposomal entrapment in order to protect the hormone from proteolytic degradation in the GI tract. In theory, to achieve the necessary degree of protection, the entrapped insulin should not penetrate or decrease the stability of the lipid bilayer. The geography of the insulin molecule within the liposome should be dependent upon the degree and type of its interactions with the lipid components. This report investigates the interactions of insulin with cholesterol, stearylamine, and various phospholipids through the use of monolayer penetration studies.

BACKGROUND

The increase in surface pressure of a lipid film with the addition of protein has been termed penetration, although the exact nature of this phenomenon remains unclear. For example, as more protein is added to the subphase, the increase in surface pressure may be attributed to an increase in the number of protein molecules at the surface, enhanced interaction between the surface components, or protein–protein interactions just below the surface, which may alter molecular orientation at the surface. Although the various mechanisms cannot always be differentiated, the results of such penetration studies are still of value in determining the criteria that affect lipid–protein interactions.

The great variation in the behavior of lipid–protein films reported in the literature can be ascribed to the wide range of physical properties of the proteins used. The unique characteristics of each protein or polypeptide prevent accurate predictions of the nature and magnitude of its interactions with lipids. Also, relative protein penetration does not show consistent results based on the type of lipid involved. Therefore, it is necessary to individually assess the effect of insulin on films of pure lipids or lipid mixtures in order to better understand their interactions.

0022-3549/83/0300-0244$01.00/0
© 1983, American Pharmaceutical Association