Pilocarpine Prodrugs II. Synthesis, Stability, Bioconversion, and Physicochemical Properties of Sequentially Labile Pilocarpine Acid Diesters

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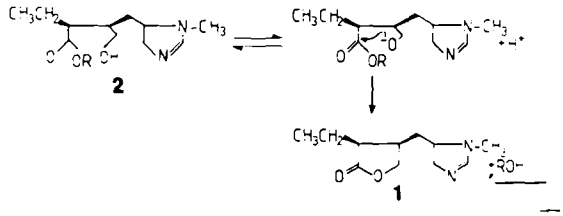
Abstract □ Various novel diesters of pilocarpic acid were synthesized and evaluated as prodrug forms for pilocarpine with the aim of improving the ocular delivery characteristics of the drug. The pilocarpic acid monoesters previously studied cyclized spontaneously to pilocarpine in aqueous solution and although they showed enhanced corneal permeability when compared with pilocarpine these monoesters suffered from poor solution stability. The present study shows that this problem can be totally overcome by blocking the free hydroxyl group in the monoesters. Diesters of pilocarpic acid were obtained by esterification of this group. Such compounds were found to possess a high stability in aqueous solution (shelf lives of more than 5 years at 20 °C were estimated) but at the same time were readily converted to pilocarpine under conditions simulating those occurring in vivo through a sequential process involving enzymatic hydrolysis of the *O*-acyl bond followed by spontaneous lactonization of the intermediate pilocarpic acid monoester. Rate data are given for the conversion of the diesters in human plasma and in various rabbit eye homogenates. The pH–solubility profile was derived for a diester and lipophilicity parameters were determined for the compounds. All diesters were markedly more lipophilic than pilocarpine and the corresponding pilocarpic acid monoesters. The results suggest that pilocarpic acid diesters may be potentially useful pilocarpine prodrugs as they combine a high solution stability with an adequate rate of conversion to pilocarpine under in vivo conditions.

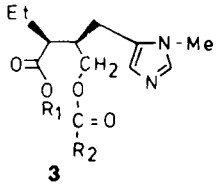
Pilocarpine (1) is widely used as a topical miotic agent for controlling the elevated intraocular pressure associated with glaucoma. However, the drug presents significant delivery problems. Its ocular bioavailability is low, the elimination of the drug from its site of action in the eye is fast, resulting in a short duration of action, and, furthermore, undesirable side effects such as myopia and miosis occur frequently as a result of systemic (noncorneal) absorption or transient peaks of high drug concentration in the eye[1] (and references cited therein).

Since these delivery problems are dependent upon the physicochemical properties (e.g. lipophilicity) of the drug, it appears likely that the delivery characteristics of pilocarpine can be improved by using the prodrug approach. To be useful a potential prodrug should exhibit a higher lipophilicity than pilocarpine in order to enable an efficient penetration through the corneal membrane, should possess sufficient aqueous solubility and stability for formulation as eyedrops, should be converted to the active parent drug within the cornea or once the corneal barrier has been passed, and should lead to a controlled release and hence prolonged duration of action of pilocarpine.

We undertook studies to develop pilocarpine prodrugs with these desirable attributes and in a foregoing paper,[1] we showed that various esters of pilocarpic acid 2 were potentially useful prodrug forms for ocular administration. The pilocarpic acid esters were found to undergo a quantitative cyclization to pilocarpine in aqueous solution (Scheme I) and



Scheme I



3

a R_1 = - CH_2  R_2 = - $CH_2CH_2CH_3$

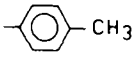
b - R_2 = - $(CH_2)_4CH_3$

c - R_2 = 

d - R_2 = 

e - R_2 = - CH_2 

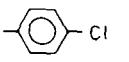
f - R_2 = 

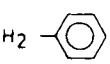
g R_1 = - CH_2  R_2 = - CH_3

h - R_2 = - $CH_2CH_2CH_3$

i - R_2 = - $C(CH_3)_3$

j - R_2 = 

k R_1 = - CH_2  R_2 = 

l R_1 = - CH_2CH_2  R_2 = 

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to be markedly more lipophilic than pilocarpine. Although such derivatives showed increased ocular bioavailability of the parent pilocarpine as a result of the greater lipophilicity, they suffered from inadequate solution stability. The rate of lactonization of the esters to pilocarpine increases proportionally with the hydroxide ion concentration in the pH range 3.5–10 and although the stability of the esters is thus much higher at an acidic pH rather than at physiological pH, ready-to-use solutions with an acceptable shelf life cannot be formulated at an acceptable pH.[1]

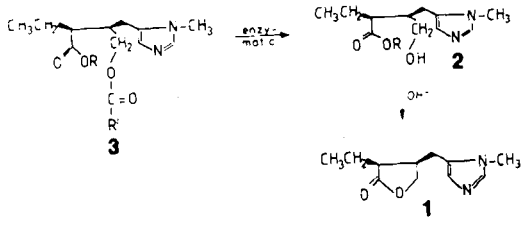
It was thought that a possible solution to this problem of obtaining a reasonable product stability might involve blocking the free hydroxyl group in the pilocarpic acid esters in such a manner that the hydroxyl group is released as a result of an enzymatic cleavage mechanism occurring in the eye. An obvious derivatization approach of the hydroxyl group is esterification since esters are susceptible to enzymatic hydrolysis. It may be envisaged that pilocarpic acid diesters 3 thus obtained would exhibit as high stability in vitro as other ester compounds but at the same time be capable of being transformed to pilocarpine in vivo through a sequential process involving enzymatic hydrolysis of the *O*-acyl bond followed by the spontaneous (nonenzymatic) lactonization of the pilocarpine acid monoester 2 (Scheme II).

The present work, part of which has been described in a preliminary manner,[2] shows that such a sequential cascade or double prodrug approach can, in fact, be used to overcome the stability problem of the pilocarpic acid monoesters. A series of pilocarpic acid diesters **3a-l** have been prepared[3] and their stability in aqueous solution and enzyme-mediated conversion to pilocarpic acid monoester investigated. Furthermore, the solubility and lipophilicity characteristics of the derivatives have been determined. In the diesters 3 studied, the R_1 groups were selected on the basis of previous findings[1] on the rate of cyclization of pilocarpic acid monoesters whereas the R_2 groups were selected so to obtain derivatives with varying enzymatic lability and lipophilicity.

Experimental Section

Melting points were determined in capillary tubes and are uncorrected. ^{1}H NMR spectra were run on a Varian 360L instrument using tetramethylsilane as the internal standard. Measurements of pH were done at the temperature of the study using a Radiometer Type PHM 26 instrument.

Synthesis of Pilocarpic Acid Diesters (3a–l)—The derivatives were synthesized by reacting pilocarpic acid monoester, prepared as previously described,[1] with the appropriate acid chloride in toluene. To a mixture of 2 mmol of pilocarpic acid monoester and anhydrous K_2CO_3 (2.5 mmol) in toluene (20 mL) were added three portions of the appropriate acid chloride (2 mmol) over a period of 24 h. A 2% aqueous solution of $NaHCO_3$ (20 mL) was added and the mixture stirred at room temperature for 3 h. The layers were separated and the organic phases were washed twice with water, dried, and evaporated under reduced pressure to give the pilocarpic acid diesters as oils. Part of each oil (1 mmol) was dissolved in ether (15 mL) and a solution of fumaric acid (174 mg; 1.5 mmol) in 2-propanol (5 mL) was added. The salts were precipitated with petroleum ether and recrystallized from 2-propanol:ether:petroleum ether. All the compounds formed salts with 1.5 equivalents of fumaric acid. Physical and analytical data for the ester fumarates are given in Table I. The ^{1}H NMR spectra of the esters were consistent with the structures.



Scheme II

In one case (*O*-butyryl pilocarpic acid 4-methylbenzyl ester, **3h**) the nitrate was prepared. The fumarate of **3h** (1.75 g; 3.0 mmol) was dissolved in a mixture of 5% aqueous $NaHCO_3$ (60 mL) and ethyl acetate (60 mL). The phases were separated and the aqueous phase extracted with ethyl acetate (60 mL). The combined organic extracts were washed with water, dried, and evaporated under reduced pressure. The oily residue was dissolved in 2-propanol (10 mL) and 4 M HNO_3 (0.8 mL) was added followed by ether (50 mL) and petroleum ether (150 mL). After standing at −20 °C for 3 days the precipitate was collected and recrystallized from ether containing 10% 2-propanol. Yield: 1.25 g (89%), m.p. 75–78 °C. Anal. ($C_{23}H_{32}N_2O_4 \cdot HNO_3$) C,H,N.

Stability Studies—The kinetics of the degradation of the pilocarpic acid diesters **3c** and **3h** were studied in aqueous buffer solutions at a constant temperature. The buffers used were hydrochloric acid, acetate (NaOAc), phosphate (KH_2PO_4), borate ($Na_2B_4O_7$), and sodium hydroxide. The total concentration of the buffers was generally 0.05 M and a constant ionic strength (μ) of 0.5 was maintained for each buffer solution by adding a calculated amount of KCl. The degradation was followed using an HPLC procedure capable of determining intact diester in the presence of degradation products. The chromatographic conditions used were: column: 250 × 4 mm, packed with LiChrosorb RP-8, particle size 7 μm (E. Merck, Darmstadt); solvent: MeOH:0.02 M KH_2PO_4 (3:1, v/v); flow rate: 1.6 mL/min; detection: 215 nm. A solvent system consisting of 60–70% v/v acetonitrile in 0.02 M phosphate of pH 7.0 was also useful and employed in several cases. The initial concentration of the derivatives in the buffer solutions was 0.05–0.1 mg/mL. At appropriate intervals, 1 mL samples of the reaction solutions were withdrawn and mixed with 1 mL of 0.1 M sodium phosphate solutions of pH 6.0. The mixture (10 μL) was then chromatographed. Quantitation of the derivatives was done from measurement of peak heights in relation to those of standards chromatographed under the same conditions. Pseudo-first-order rate constants for the degradation were determined from the slopes of linear plots of the logarithm of residual pilocarpic acid diester against time.

Analysis of degradation products formed (pilocarpic and isopilocarpic acid) in alkaline aqueous solution was performed by HPLC with a modification of a previously described method[4] and used the following conditions: column: 250 × 4 mm, packed with LiChrosorb RP-18, particle size 5 μm; solvent: MeOH:0.05 M phosphate buffer of pH 2.5 (1:19 v/v); flow rate: 1.6 mL/min; detection: 215 nm.

The hydrolysis of the other pilocarpic acid diesters was examined in dilute sodium hydroxide solutions (0.005–0.02 M; μ = 0.02) at 37 °C. The reactions were followed by HPLC analysis for remaining diesters as described above.

Conversion Kinetics in Human Plasma—The conversion of the diester derivatives was studied in 0.01 M phosphate buffer of pH 7.40 containing 75% human plasma at 37 °C. Initial concentrations of the compounds were 0.03–0.06 mg/mL. At appropriate times samples of 250 μL were withdrawn and added to 1000 μL of ethanol in order to deproteinize the plasma. After mixing and centrifugation for 3 min, 10–20 μL of the clear supernatant was analyzed for remaining diester and monoester formed using the HPLC procedure described

Table I—Physical Data of Various Pilocarpic Acid Diesters 3

Compound	Yield, %	mp, °C[a]	Formula[b]
3a	52	63–65	$C_{28}H_{36}N_2O_{10}$
3b	45	60–62	$C_{30}H_{40}N_2O_{10}$
3c	65	88–90	$C_{31}H_{34}N_2O_{10}$
3d	69	77–78.5	$C_{31}H_{33}ClN_2O_{10}$
3e	44	63–65	$C_{32}H_{36}N_2O_{10}$
3f	51	71–75	$C_{30}H_{33}N_3O_{10} \cdot 0.5H_2O$
3g	85	80–81	$C_{27}H_{34}N_2O_{10}$
3h	65	89–91	$C_{29}H_{38}N_2O_{10}$
3i	53	123–125	$C_{30}H_{40}N_2O_{10}$
3j	65	86–87	$C_{32}H_{36}N_2O_{10}$
3k	61	109–111	$C_{31}H_{33}ClN_2O_{10}$
3l	34	73–77	$C_{32}H_{36}N_2O_{10}$

[a] All compounds were recrystallized from 2-propanol:ether:petroleum ether. [b] Salts with 1.5 equiv fumaric acid.

above with MeOH:0.02 M KH_2PO_4 (3:1 v/v, %) as the eluant. Under these conditions the diesters were well separated from the corresponding monoesters and both could readily be determined. While pilocarpine also could be determined using these conditions in degradation studies in pure buffer solutions, it was not well resolved from the solvent front in case of analysis of the plasma solutions. Instead, pilocarpine produced in the plasma solutions was determined by the HPLC procedure previously described.[5] This procedure allows the simultaneous separation and determination of pilocarpine and its degradation products (isopilocarpine, pilocarpic acid and isopilocarpic acid). Pilocarpic acid mono and diesters were retained on the column in this procedure.

Hydrolysis in Ocular Tissue Homogenates—The ocular tissue homogenates were prepared from the eyes of adult male New Zealand white rabbits weighing 2.2–3.0 kg. They were sacrificed by a rapid injection of sodium pentobarbital (50 mg/mL) into a marginal ear vein. The corneal surfaces were rinsed with saline, blotted dry and the aqueous humor was aspirated from each eye with a 25 gauge × 1.59 cm needle attached to a 1-mL tuberculin syringe. The cornea, lens, and iris/ciliary body[6] was then removed from each eye with a surgical scalpel.

The aqueous humor and all tissues were immediately transferred to cold, preweighed 18 × 120 mm heavy-walled glass centrifuge tubes with screw caps. The samples were maintained at 0–5 °C. The tissue weights were determined and enough cold isotonic pH 7.40 phosphate buffer (0.067 M) was added to give 20% w/v solutions. The aqueous humor was not diluted. The tissues were then homogenized with a motor driven tissue grinder for 30 s while maintaining the temperature at 0–5 °C. The tubes were then capped and the samples were centrifuged at 4 °C and 7800 × *g* for 20 min. The supernatants were saved for enzymatic hydrolysis studies. The protein content of each supernatant was determined using a protein-dye binding assay (Bio-Rad Laboratories, Richmond, CA) with rabbit serum albumin as the reference. Tissues from more than one rabbit were not pooled, but each hydrolytic study was repeated with the supernatants from at least two other animals.

Solutions of the prodrugs or the model substrate, α-naphthyl acetate, were prepared at 3×10^{-5} M by dissolving the appropriate amount of the compound in 2.5 mL of ethanol, and bringing the volume to 25 mL with isotonic pH 7.40 phosphate buffer (0.067 M). The ethanol was used for solubility reasons. Where possible, the effect of the ethanol on the esterase activity was evaluated by determining the enzymatic rate in the presence of 10%, 5%, and 0% ethanol. The results showed a ten- to twenty-fold reduction in activity in the presence of 10% ethanol. The greatest reduction occurred with the iris preparation, then with the cornea preparation, and then with the lens preparation. For a given tissue, the percent reduction was the same for all compounds.

A 450-μL aliquot of a substrate solution was placed into a 0.5 dram vial maintained at 34 °C. The solution was allowed to equilibrate to the temperature and then 50 μL of a tissue supernatant as prepared above was added. At preselected times, 50 μL aliquots of the contents of the vial were taken, diluted with 50 μL of 0.1 M HCl and immediately analyzed.

For the K_m and V_{max} determinations, five solutions of each prodrug evaluated were prepared in 10% ethanol/buffer to give a concentration range of approximately 2×10^{-5} M to 2×10^{-4} M. A 25-μL aliquot of an iris supernatant was added to 225 μL of each of the prodrug solutions maintained at 34 °C. The initial hydrolytic rates were determined by taking 50-μL samples at times of 0, 1, and 2 min following the additions. The samples were diluted with an equal volume of 0.1 M HCl and analyzed.

Determination of α-naphthyl acetate and its hydrolytic product, α-naphthol was performed by reversed-phase HPLC with UV detection at 254 nm. The HPLC conditions were: column: 150 × 4.6 mm, packed with MOS Hypersil (C_8), particle size 5 μM; solvent: methanol:water (65:35 v/v); flow rate 1.5 mL/min. For the prodrugs, analysis was performed by ion-exchange HPLC with UV detection at 214 nm. The chromatographic conditions for the prodrugs were: column: 250 × 4.6 mm Whatman Partisil PXS 10/25 strong cation exchange; solvent: methanol:0.075 M KH_2PO_4 (17:83 v/v); flow rate: 1.5 mL/min. This allowed the separation and quantitation of the diesters, monoesters and pilocarpine on the same system. Direct injection of the supernatants required the use of a precolumn filter (Uptight Precolumn Filter, Upchurch Scientific, WA) with replaceable frits in both systems.

Determination of Lipophilicity Parameters—The apparent partition coefficients of the diesters were determined in octanol-buffer systems at 20–22 °C. The aqueous phase was 0.05 M acetate or phosphate buffer solutions of pH 4–6. The buffer solutions and octanol were mutually saturated at 20–22 °C before use. The compounds were dissolved in the aqueous buffer phase (~0.05 mg/mL) and the octanol-water mixtures were shaken for several hours to reach a distribution equilibrium. The volumes of each phase were chosen so that the solute concentration in the aqueous phase, before and after distribution could readily be measured using the aforementioned HPLC procedure. The partition coefficients (*P*) were calculated from eq. 1:

$$P = \left(\frac{C_i - C_w}{C_w}\right)\left(\frac{V_w}{V_o}\right) \quad (1)$$

where C_i and C_w represent the solute concentrations in the aqueous buffer phase before and after distribution, respectively; V_w represents the volume of the aqueous and V_o the volume of the octanol phase. In some cases the solute concentration in the octanol phase was also determined, the *P* values thus obtained being in good agreement with those derived on basis of eq. 1. For each compound, determinations were carried out in triplicate, and the *P* values thereby obtained were reproducible to within ±5%.

The lipophilicity of the derivatives was also evaluated by means of reversed-phase HPLC, as described previously for pilocarpic acid monoesters.[1] In this method the capacity factor (*k'*) of a solute is taken as a measure for the relative lipophilicity:

$$k' = (t_R - t_o)/t_o \quad (2)$$

where t_R is the retention time of the solute and t_o is the elution time of the solvent.

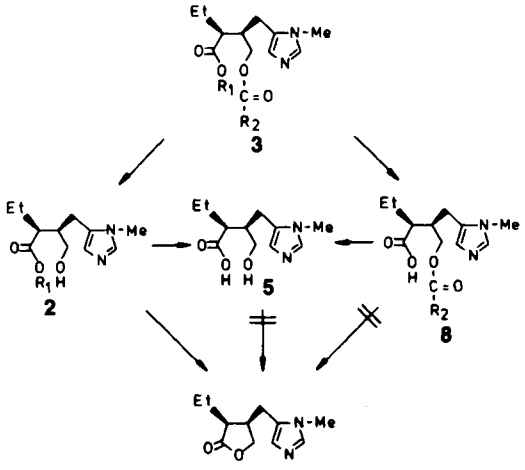
pH-Solubility Profile—The pH–solubility profile of *O*-butyryl pilocarpic acid 4-methylbenzyl ester nitrate salt (**3h**) was determined by the phase–solubility technique at 23 °C. A 1.6-g sample of **3h** was dissolved in 20 mL of water. The pH of the solution was 3.25. The solution was stirred at 23 °C and 0.1 M or 2 M NaOH was added dropwise. After each addition the mixture was stirred for 1 h, the pH was recorded and an aliquot of 1 mL was withdrawn. Each aliquot was centrifuged and the clear supernatant was analyzed by HPLC as described above upon appropriate dilution with water, the final dilution (1:1) being made with 0.1 M phosphate buffer of pH 6.0. This dilution with buffer was necessary to ensure a reproducible peak height of the ester. For unknown reasons, perhaps due to column adsorption phenomena, the peak height of the lipophilic diesters was dependent on the composition of the solution analyzed. In a given buffer the peak height was highly reproducible, the day-to-day variation being <2%.

Quantitation of **3h** was done from measurement of the peak height in relation to those of standard solutions, made with 0.05 M phosphate buffer of pH 6.0, chromatographed under the same conditions. It was shown that the solubility did not change significantly if the equilibration of solution was continued for more than 1 h after each addition of NaOH. It was further ensured by HPLC analysis that **3h** was completely stable during the solubility determination.

Results and Discussion

Bioconversion of Pilocarpic Acid Diesters—For the evaluation of the pilocarpic acid diesters **3** as potential prodrugs of pilocarpine it is important to ascertain whether enzymes, especially those present in ocular tissues,[7] would be able to cleave the diester into the pilocarpic acid monoester which subsequently can cyclize nonenzymatically to pilocarpine. The two ester groups in **3** may both be thought to undergo enzymatic hydrolysis but pilocarpic acid monoester **2** and, hence, pilocarpine will only be formed as a result of enzymatic hydrolysis of the R_2 ester moiety. Hydrolysis of the R_1 ester group would give *O*-acyl pilocarpic acid **8** whereas hydrolysis of both ester groups results in the formation of pilocarpic acid (**5**) (Scheme III). Neither of these compounds is able to cyclize to pilocarpine at physiological pH.

Hydrolysis in Human Plasma—The pilocarpic acid diesters were initially studied in human plasma solutions since plasma is rich in various esterases and, furthermore, plasma



Scheme III

has been shown to be a good model of ocular tissue enzymes for ester hydrolysis.[8-10] When incubated in 75% human plasma solution (pH 7.4) at 37 °C the diesters were found to degrade very rapidly as compared with the extremely high stability of the compounds in pure buffer solutions of pH 7.4 (Table II). The degradation followed strict first-order kinetics under the experimental conditions used (Fig. 1). By analyzing the reaction solutions for the corresponding pilocarpic acid monoester and pilocarpine, time-courses for the various species as those shown in Fig. 2 were observed. As can be seen the diester is hydrolyzed to pilocarpic acid monoester 2j which subsequently undergoes ring closure with formation of pilocarpine. In Fig. 2 the sum of concentrations of the diester 3j, the intermediate pilocarpic acid 4-methylbenzyl ester (2j) and the final product pilocarpine is 100 ± 3%, thus demonstrating the quantitative nature of the two steps shown in Scheme II.

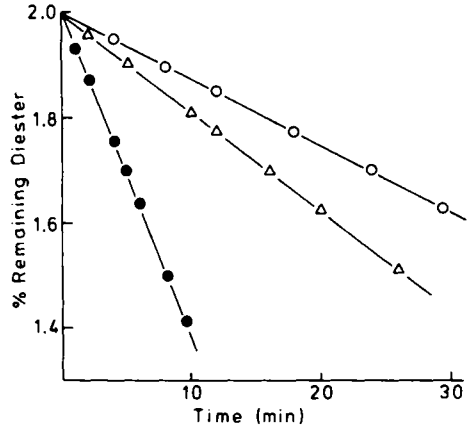


Figure 1—*First-order plots for the hydrolysis of the pilocarpic acid diesters* **3g** (○), **3h** (●) *and* **3j** (△) *in 75% human plasma solutions (pH 7.40) at 37 °C.*

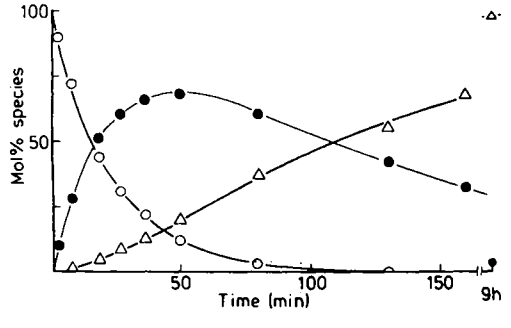


Figure 2—*Time course for O-benzoyl pilocarpic acid 4-methylbenzyl ester* (**3j**) (○), *pilocarpic acid 4-methylbenzyl ester* (●) and pilocarpine (△) *during incubation of compound* **3j** *in 75% human plasma (pH 7.40) at 37 °C. The curves are calculated from eqs. 3–5.*

The time-courses for the various species may be described by the following equations.[11]

$$[\text{Diester}]_t = [\text{Diester}]_o \cdot e^{-k_d t} \quad (3)$$

$$[\text{Monoester}]_t = [\text{Diester}]_o \frac{k_f}{k_c - k_f} (e^{-k_f t} - e^{-k_c t}) \quad (4)$$

$$[\text{Pilocarpine}]_t = [\text{Diester}]_o \left[1 + \frac{1}{k_f - k_c}\left(k_c e^{-k_f t} - k_f e^{-k_c t}\right)\right] \quad (5)$$

where k_f and k_c are pseudo-first-order rate constants for the formation and cyclization of the monoester, respectively, and k_d is a pseudo-first-order rate constant for the overall degradation of the diester. In Fig. 2 the curves were constructed using the following values of the rate constants:

$$k_d = 0.044 \text{ min}^{-1}$$

$$k_f = 0.043 \text{ min}^{-1}$$

$$k_c = 0.0087 \text{ min}^{-1}$$

The close agreement between k_d and k_f is further evidence for the quantitative course of the diester→monoester conversion. The half-time of conversion of the 4-methylbenzyl pilocarpic acid monoester to pilocarpine is 80 min ($0.693/k_c$) which is similar to the value (77 min) observed[1] in pure buffer solutions. Both the rate and extent of conversion of pilocarpic acid monoesters to pilocarpine have previously been shown to be unaffected by human plasma.[1]

Inspection of the data in Table II shows that most diesters are rapidly hydrolyzed by plasma enzymes. Only the *O*-pivaloyl ester 3i is hydrolyzed rather slowly which may be ascribed to the bulky *tert*-butyl side chain. An explanation of the specificity of the enzymatic ester hydrolysis, i.e. that the

Table II—Rate Data for the Hydrolysis of Various Pilocarpic Acid Diesters at 37 °C

Pilocarpic Acid Diester	k_{OH}, $M^{-1} \cdot min^{-1}$ [a]	$t_{1/2}$, h pH 7.4 [b]	$t_{1/2}$, min 75% human plasma
3a	3.5	5.5×10^3	3
3b	3.3	5.8×10^3	4
3c	3.8	5.1×10^3	12
3d	14.6	1.3×10^2	25
3e	21.7	8.8×10^2	4
3f	44.4	4.3×10^2	6
3g	12.6	1.5×10^2	24
3h	3.5	5.5×10^3	5
3i	0.26	7.4×10^4	330
3j	4.0	4.8×10^3	16
3k	3.4	5.6×10^3	17
3l	3.8	5.1×10^3	15

[a] Hydroxide ion catalytic rate constant ($= k_{obs}/a_{OH}$) for the disappearance of diester in alkaline aqueous solution ($\mu = 0.02$). [b] Calculated on the basis of k_{OH}. The rate of degradation is proportional to the hydroxide-ion activity at pH > 7 (see Fig. 8).

diesters are exclusively attacked at the R_2 ester moiety, may be that the acyl portion of the R_1 ester is more sterically hindered than the acyl portion of the R_2 ester (except in case of the slowly hydrolyzing compound **3i** for which no product analysis was performed). Decreased enzymatic catalysis of ester hydrolysis with increasing bulkiness of the acyl ester moiety has commonly been observed.[12]

Hydrolysis in Ocular Tissue Homogenates—Use of the model substrate, α-naphthyl acetate, verified the presence of general esterases in the ocular supernatants. In fact, a 10-fold reduction in the concentration of the supernatants was required in order to slow the reaction to an easily measurable rate. An example of the activity seen is given in Fig. 3. As compared to pH 7.40 phosphate buffer, the esterases present in the ocular supernatants caused a rapid hydrolysis of the α-naphthyl acetate. HPLC analysis verified a quantitative hydrolysis of the ester to the corresponding alcohol, α-naphthol.

Several pilocarpic acid monoesters **2** were evaluated as substrates for the ocular esterases with equivalent results for all. As shown in Fig. 4, where the rate of loss of pilocarpic acid benzyl ester in the presence of the eye tissue homogenate supernatants is compared to the rate of loss in the presence of only buffer at pH 7.40, no appreciable difference is observed. The supernatants used in this study were also evaluated with the model substrate and substantial esterase activity was shown to be present. Therefore, as found in the plasma studies,[1] the rate of enzymatic hydrolysis of the pilocarpic acid monoesters is negligible when compared to the rate of the apparent hydroxide ion-catalyzed ring closure. Since no measurable enzymatic catalysis was observed for the slowly cyclizing pilocarpic acid hexyl ester ($t_{1/2}$ at pH 7.40 is 18.4 h^{-1}), the pilocarpic acid monoesters **2** are indeed poor substrates for the enzyme systems present in the ocular tissues.

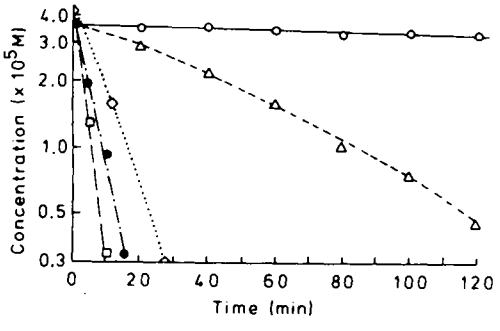


Figure 3—*First-order plots for the hydrolysis of α-naphthyl acetate at 34 °C in the presence of ocular tissue esterases. Key: (○) pH 7.40 buffer; (△) aqueous humor; (◇) lens; (●) cornea; (□) iris–ciliary body.*

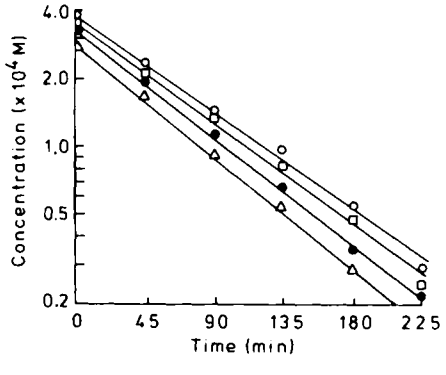


Figure 4—*First-order plots for the hydrolysis of pilocarpic acid benzyl ester at 34 °C in the presence of ocular tissue esterases. Key: (○) pH 7.40 buffer; (△) aqueous humor; (●) cornea; (□) iris–ciliary body.*

The pilocarpic acid diesters **3**, however, showed substantial enzymatic hydrolysis by the ocular esterases, verifying the results obtained from the plasma studies. Selective cleavage of the R_2 ester moiety was again seen with quantitative formation of pilocarpic acid monoester and subsequent spontaneous (uncatalyzed) cyclization of monoester to pilocarpine. Figure 5 is indicative of the esterase activity observed toward the diesters. It shows the loss over time of compound **3h** in the presence of aqueous humor, and supernatants of lens, cornea, and iris/ciliary body tissue homogenates. Since the supernatants contain differing amounts of protein, a direct comparison of the reaction rates is inappropriate. Although the exact types of enzymes present in each tissue and their distribution within the protein present are unknown, a measure of the amount of total protein in each supernatant allowed the calculation of relative activities between tissues. The initial rates, so determined, for the enzymatic hydrolysis of compound **3h** are compared in Fig. 6. The iris/ciliary body showed the greatest activity, followed by the cornea and then the lens and aqueous humor. These results are in agreement with those obtained by Lee[13] with respect to rank order but show three to eight-fold less activity than toward the model substrate used in his work.

For each tissue, the rates of enzymatic hydrolysis differed as the R_2 moiety was altered. A correlation was attempted between the pseudo-first-order rate constant for enzymatic hydrolysis and the octanol-buffer partition coefficient of the

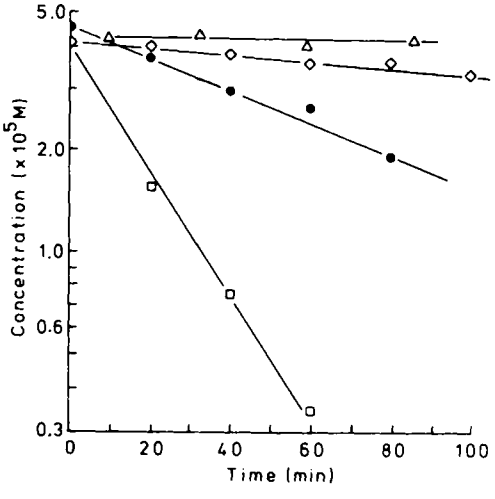


Figure 5—*First-order plots for the hydrolysis of O-butyryl pilocarpic acid 4-methylbenzyl ester* (**3h**) *at 34 °C in the presence of ocular tissue esterases. Key: (△) aqueous humor; (◇) lens; (●) cornea; (□) iris–ciliary body.*

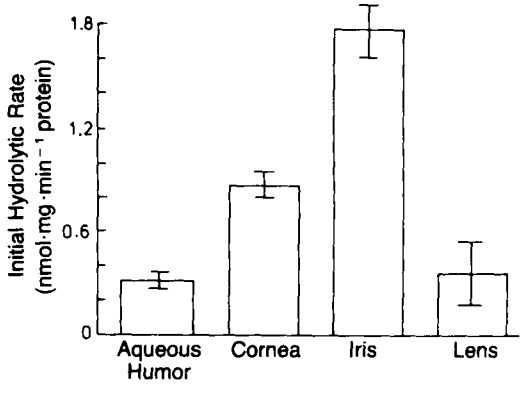


Figure 6—*Mixed esterase activity towards the O-butyryl pilocarpic acid 4-methylbenzyl ester* (**3h**) *for various ocular tissues at 34 °C. (Error is SEM, n = 3).*

esters but variations in the R_1 moiety altered the partitioning behaviour with no apparent effect on the enzymatic rate. The observed partition coefficient was therefore replaced in the correlation with the Hansch hydrophobic substituent parameter π[14] for the R_2 substituents. With this approach, the hydrophobic character of only the group undergoing cleavage is used. The results of this correlation are shown in Fig. 7.

Two different correlations are seen, one for aromatic groups and one for aliphatic groups used as R_2 moieties. The observed pseudo-first-order enzymatic rate constants increase with increasing π for the aliphatic chains and decrease with increasing π for the aromatic moieties. This difference may be due to either a difference in the ability of the two groups to fit the enzymatic site or to different enzyme types being responsible for the hydrolysis of the two groups (e.g. carboxylic ester hydrolase and aryl ester hydrolase). There appears to be no effect on the rate constant when the R_1 moiety is altered, as evidenced by nearly equivalent values for compounds **3a** and **3h** as well as for **3c** and **3j**, where the R_2 moieties are the same and the R_1 groups are different. The trends shown in Fig. 7 also appeared for the corneal and the lens data.

In order to provide a clearer understanding of these findings, the Michaelis-Menten parameters V_{max} and K_m were determined for the mixed esterase system in the iris/ciliary body where the substrates are compounds with R_2 = alkyl. The rate data obtained were plotted as the velocity versus the velocity divided by the substrate concentration.[15] The results were linear plots, supporting the use of Michaelis-Menten kinetics to describe the enzymatic reactions. The parameters determined from the plots are given in Table III.

The K_m values are seen to decrease slightly and the V_{max} values increase substantially with increasing chain length within the compounds examined. Similar results were obtained by Webb and co-workers[16] in a study on horse liver carboxylesterase-catalyzed ester hydrolysis where the effects of chain length on reactivity (V_{max}) and affinity ($1/K_m$) were investigated.

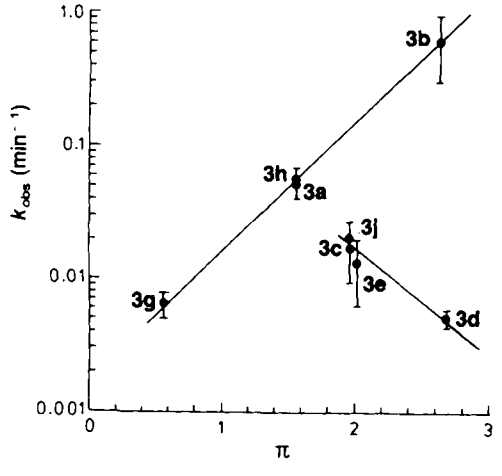


Figure 7—*A plot of the logarithm of the observed pseudo-first-order rate constants for hydrolysis of pilocarpic acid diesters* **3** *to the corresponding monoesters* **2** *by iris–ciliary body esterases versus the hydrophobic substituent parameter* π *for the* R_2 *group.*

Table III—Michaelis-Menten Parameters for the Hydrolysis of 3g, 3h and 3b by the Mixed Esterases in the Iris/Ciliary Body Preparation

Pilocarpic Acid Diester	$K_m \times 10^5$, M[b]	V_{max}[a]
3g	4.11	1.48
3h	4.01	8.74
3b	3.09	54.30

[a] Nanomoles of substrate hydrolyzed per milligram of protein per minute. [b] Carried out at 34 °C.

Stability of Pilocarpic Acid Diesters in Aqueous Solution—In order to predict the stability of the pilocarpic acid diesters under conditions similar to those encountered for storage of e.g. aqueous eye-drop formulations, the decomposition kinetics of the diesters **3c** and **3h** was examined in aqueous solution as a function of pH and temperature. Under the experimental conditions used the degradation of the compounds followed strict first-order kinetics. No significant catalysis by the buffers used to maintain constant pH was observed in buffer concentrations less than 0.05 M.

The influence of pH on the rates of degradation at 70 °C is shown in Fig. 8 in which the logarithm of the observed pseudo-first-order rate constants (k_{obs}) is plotted against pH. The shape of these profiles indicates that the diesters are subject to specific acid- and base-catalyzed hydrolysis as well as a water catalyzed reaction (especially compound **3c**). The profiles show a curvature at pH values around the pK_a value of the compounds which indicates that the protonated form of the molecules undergoes hydroxide ion-catalyzed hydrolysis at a rate that is greater than that of the free base form. The rate equation accounting for the pH dependence of k_{obs} may be formulated as:

$$k_{obs} = k_H a_H \frac{a_H}{a_H + K_a} + k_o \frac{a_H}{a_H + K_a} + k'_{OH} a_{OH} \frac{a_H}{a_H + K_a} + k_{OH} a_{OH} \frac{K_a}{a_H + K_a} \quad (6)$$

where a_H and a_{OH} refer to the hydrogen-ion and hydroxide-ion activity, respectively, $a_H/(a_H + K_a)$ and $K_a/(a_H + K_a)$ are the fractions of the compounds in the protonated and unprotonated form, respectively, and K_a is the apparent ionization constant of the compounds. The rate constants k_H and k_o refer to hydrogen ion catalyzed and water-catalyzed hydrolysis of protonated species, respectively, while k'_{OH} and k_{OH} are the second-order rate constants for the hydroxide ion catalyzed hydrolysis of the protonated and unprotonated species, respectively. The various rate and ionization constants derived at 70 °C are listed in Table IV. In Fig. 8 the solid curves drawn were constructed from these constants and eq. 6, and the good agreement observed between the calculated and

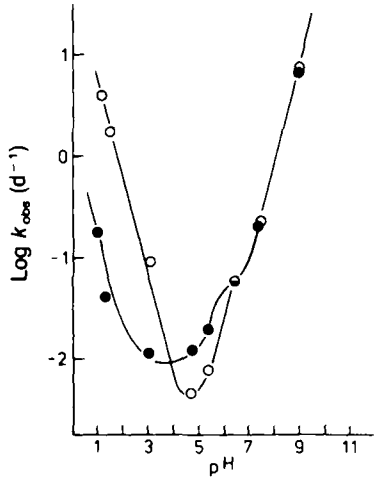


Figure 8—*pH–Rate profiles for the degradation of the pilocarpic acid diesters* **3c** (●) *and* **3h** (○) *in aqueous solution* (μ = *0.5) at 70 °C.*

Table IV—Rate Data for the Hydrolysis of the Pilocarpic Acid Diesters 3c and 3h in Aqueous Solution (μ = 0.5) at 70 °C

Compound	k_H, $M^{-1}\cdot h^{-1}$	k_0, h^{-1}	k'_{OH}, $M^{-1}\cdot h^{-1}$	k_{OH}, $M^{-1}\cdot h^{-1}$	pK_a[a]
3c	7.8×10^{-2}	3.3×10^{-4}	1.9×10^{4}	2.3×10^{3}	6.3
3h	2.34	0.9×10^{-4}	7.7×10^{3}	2.2×10^{3}	6.3

[a] At 20 °C the pK_a values were determined to be 7.0. The lower values found at 70 °C are as expected for weak bases like the imidazole derivatives **3c** and **3h**.

experimental data demonstrates that eq. 3 adequately accounts for the degradation of the pilocarpic acid diesters. At 70 °C maximum stability of **3h** in aqueous solution occurs at pH 4.2–5.0 whereas **3c** is most stable at pH 3.5–4.5. It is of interest to note that for compound **3c** a water-catalyzed or spontaneous reaction is a major degradation pathway at pH 3–5.

The rates of degradation of compound **3c** in a 0.05 M acetate buffer of pH 4.6 were determined at 70, 80 and 90 °C. By plotting the rate constants obtained according to the Arrhenius equation, an energy of activation of 27.9 kcal/mol was obtained. On the basis of this value and the rate constant observed at e.g. 70 °C it is possible to estimate the shelf life of aqueous solutions of compound **3c** at pH 4.6 at various temperatures. Defining the shelf life as the time required to degrade 10% of the compound ($t_{10\%}$) the calculations show that shelf lives of ~8 and 21 years are achieved at 25 and 20 °C, respectively. Thus, the compound can be formulated as highly stable, ready-to-use solutions at pH 3–5. Interestingly, the stability of this prodrug exceeds even that of pilocarpine in aqueous solution as assessed on basis of described stability data for pilocarpine.[17–19]

Since more than one reaction is important in the degradation of compound **3h** at pH 4–5, prediction of its stability at room temperature was made in a way different from that used for compound **3c**. The rates of hydrolysis of compound **3h** in acidic and alkaline buffer solutions were determined at various temperatures (40–70 °C) and the specific acid and base catalytic rate constants were obtained at 20 °C from the Arrhenius equation. On this basis the pH–rate profile for **3h** was constructed at 20 °C (Fig. 9). It can be seen from the figure that the maximal stability at 20 °C occurs at pH 5.0–5.5 and that a shelf life of ≥2 years is achieved within the pH-range 4.4–6.2. A shelf life of about 5 years can be predicted for solutions of pH 5–5.6.

Mechanism of Degradation—The theoretical pathways of degradation of a pilocarpic acid diester **3** are shown in Scheme IV. Compound **3** may degrade by three parallel reactions: hydrolysis of the R_2-ester bond (k_1), hydrolysis of the R_1-ester bond (k_2) and isomerization (k_3). The isomer **4** may possibly have the same retention time as **3** in HPLC and if formed, the stability data given above may be overestimated. The end product of the tentative isomerization reaction would be isopilocarpic acid (**6**) whereas the other two hydrolysis reactions yield pilocarpic acid (**5**). To determine whether any base-catalyzed isomerization (analogous to the isomerization of pilocarpine to isopilocarpine[5,19–22]) occurs simultaneously with hydrolysis of the ester moieties the products of degradation of compound **3c** were examined.

Alkaline aqueous solutions (pH 10–13) of **3c** were kept at 20 °C. When all **3c** had disappeared the solutions were analyzed for pilocarpic and isopilocarpic acid by a specific HPLC procedure. In alkaline solutions pilocarpine and isopilocarpine were determined to be six times more labile than **3c** and, therefore, these compounds disappear rapidly following their formation from **3c**. It was found that the two carpic acids were formed in quantitative amounts on a molar basis: 88% pilocarpic acid and 12% isopilocarpic acid. In alkaline solutions pilocarpine degrades both to pilocarpic acid and isopilocarpic acid (the latter via isopilocarpine), the relative importance of hydrolysis and isomerization being dependent on the temperature.[5] Under similar conditions as those used for compound **3c**, pilocarpine was found to degrade to 89% pilocarpic acid and 11% isopilocarpic acid, which is in agreement with previous results.[5]

These data provide strong evidence that isomerization of **3** plays no significant role in the overall degradation. Compound **3c** degrades to yield pilocarpic and isopilocarpic acid in amounts quite similar to those formed from pilocarpine when this was degraded under similar conditions. If isomerization of **3** were a significant process, greater amounts of isopilocarpic acid should have been formed at the expense of pilocarpic acid.

Concerning the relative importance of the reactions k_1 and k_2, the former reaction must be the predominant degradation route. This is apparent by inspection of the k_{OH} values for the degradation of the various diesters given in Table II. It is seen that the k_{OH} values are predominantly dependent on the R_2 group, cf. e.g. the *O*-acetyl, *O*-butyryl, *O*-pivaloyl and *O*-

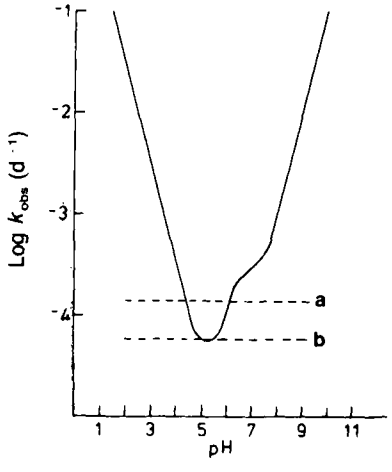
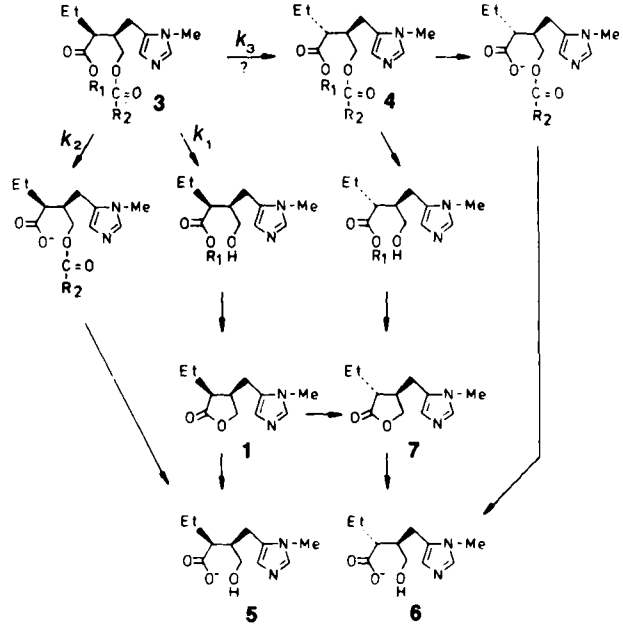


Figure 9—*Estimated pH–rate profile for the degradation of compound* **3h** *in aqueous solution (μ = 0.5) at 20 °C. Key: (a) $t_{10\%}$ = 2 years; (b) $t_{10\%}$ = 5 years.*



Scheme IV

benzoyl derivatives (3g–j) of pilocarpic acid 4-methylbenzyl ester. If the k_2-reaction were important one should expect compound 3i to degrade much faster than actually observed. Further supporting evidence for the predominance of the k_1-reaction comes from the fact that the end products of alkaline degradation of 3c are quantitatively similar to those formed from pilocarpine which can only be formed along the k_1-reaction pathway. It appears that $k_1/k_2 > 20$. Thus, it can be concluded that the predominant route of degradation of the diesters involves hydrolysis of the R_2 ester moiety to yield pilocarpic acid monoester. This compound has only a transistory existence and is rapidly cyclized to pilocarpine[1] which then degrades to pilocarpic and isopilocarpic acid.

The Lipophilicity of the Pilocarpic Acid Diesters—The apparent partition or distribution coefficients (P) for the diesters were measured using the widely used octanol–water system. The primary interest was to know the lipophilicity at physiological pH but as the partition coefficients were very high and thus not easily determinable at pH 7.4, P values were determined using aqueous buffers of pH 4–6. Assuming that the protonated forms of the diesters do not partition significantly relative to the free base forms the partition coefficients at pH 7.4 ($P_{pH\ 7.4}$) can be calculated from the P values measured at other pH values (P_{pH}) by means of the following equation:

$$P_{pH\ 7.4} = P_{pH}\frac{1 + 10^{(pK_a - pH)}}{1 + 10^{(pK_a - 7.4)}} \quad (7)$$

Equation 7 was derived by combining the following two equations:

$$P_B = P_{pH}(1 + 10^{(pK_a - pH)}) \quad (8)$$

$$P_{pH\ 7.4} = P_B/(1 + 10^{(pK_a - 7.4)}) \quad (9)$$

where P_B represents the partition coefficient of the unionized form.

In these calculations a pK_a value of 7.0 at room temperature (see later) was used for all diesters. The validity of this procedure to estimate the partition coefficients at pH 7.4 was supported by the constancy observed for $P_{pH\ 7.4}$ calculated from P_{pH} values obtained at different pH values. An example is shown in Table V. The values determined for log $P_{pH\ 7.4}$ are listed in Table VI. The log P values are mutually in good agreement with values calculated on basis of π substituent values.[14] For example, on going from the O-acetyl ester 3g to the O-butyryl ester 3h, log P increases by 1.03 which corresponds to the π value for two methylene groups (1.00). Thus it is possible to predict the partition coefficients of other pilocarpic acid diesters on the basis of the additive substituent principle.

As expected, the esterification of the hydroxyl group in the monoesters gives more lipophilic compounds (diesters). By comparing the log P values for the diesters with those previously described[1] for the corresponding monoesters, it can thus be seen that O-acetylation leads to an increase in log P of 0.85 whereas O-benzoylation affords an increase in log P of 2.4. These figures are also in good agreement with those predicted from π substituent constants.[14]

Since pilocarpine itself has a log P value of −0.15 at pH 7.4 it can be seen that the diester prodrugs afford a 10^3–10^5 times increase in lipophilicity in terms of octanol–pH 7.4 buffer partition coefficients.

The lipophilicity of the derivatives was also evaluated by means of HPLC capacity factors (k'). With MeOH:0.02 M KH_2PO_4 (pH ~ 4.5) (1:3 v/v) as mobile phase the diesters showed the k' values given in Table VI. As has been observed for many different types of compounds[23,24] (and references cited therein) a linear relationship exists between log k' and log P for the diesters. As seen in Fig. 10 the relationship (log P = 4.20 log k' + 2.60) not only fits the data for the diesters but also includes pilocarpine and various pilocarpine acid monoesters. Using this relationship it is simple to predict with great confidence the log P values for other pilocarpic acid derivatives.

pH-Solubility Profile for Compound 3h—The aqueous solubility of compound 3h (nitrate salt) was determined at 23 °C as a function of pH. When 1.6 g of the compound was mixed with 20 mL of water a clear solution was obtained, and the resulting pH was 3.25. This implies that the solubility of the nitrate salt in water is >8% w/v. By addition of 0.1 M NaOH until a pH of 4.4 was reached the solution remained clear for several days. At pH ≥4.5 the solution turned cloudy due to precipitation of the free base. Upon further addition of

Table V—Observed and Calculated Partition Coefficients for Compound 3h between Octanol and Aqueous Buffers

pH of Buffer	log P^a	log P^b pH 7.4
5.20	2.55	4.21
5.00	2.33	4.19
4.50	1.80	4.15
4.00	1.36	4.21

[a] Observed values. [b] Values calculated on basis of eq. 7 and a pK_a value of 7.00.

Table VI—Partition Coefficients (P) and Chromatographic Capacity Factors (k') of Pilocarpine and Pilocarpic Acid Diesters

Compound	log P^a	k'
Pilocarpine[b]	−0.15	0.22
3a	3.63	2.01
3b	4.60	3.02
3c	4.22	2.55
3d	4.93	3.63
3e	3.85	2.09
3f	2.90	1.20
3g	3.16	1.45
3h	4.19	2.50
3i	4.40	2.66
3j	4.70	3.39
3k	4.75	3.43
3l	4.60	3.05

[a] Partition coefficient between octanol and aqueous buffer of pH 7.4. [b] From a previous study (ref. 1).

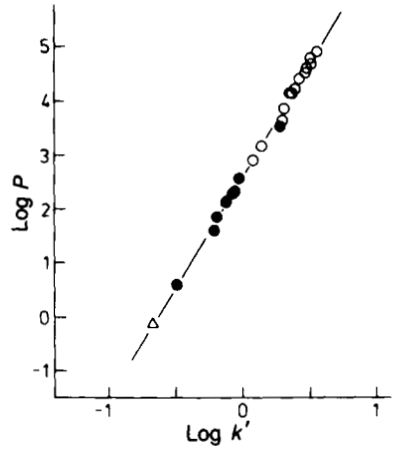


Figure 10—*Plot of log k' versus log P (at pH 7.4) for pilocarpine (△), various pilocarpine acid monoesters (●) and pilocarpic acid diesters (○). The values for the latter are taken from Table VI whereas the values for the other compounds are from a previous study (ref. 1).*

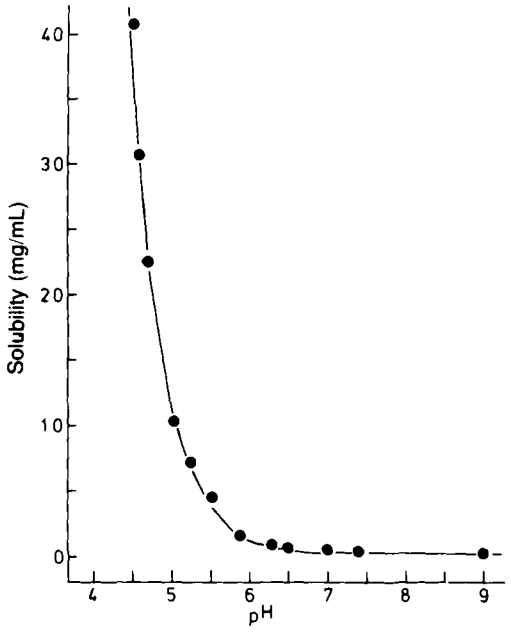


Figure 11—*Aqueous solubility–pH profile for compound* **3h** *at 23 °C. The points are experimental while the curve is calculated from eq. 10.*

NaOH the free base separated as an oil. The pH-solubility profile obtained is shown in Fig. 11. In the pH-range studied

solubility (S_T) of the compound. Thus, the following equation is valid:

$$S_T = [B]_s \frac{a_H + K_a}{K_a} \quad (10)$$

where $[B]_s$ is the concentration of the free base in the saturated solution, a_H is the hydrogen-ion activity and K_a is the ionization constant of protonated **3h**. Rearranging eq. 10 gives:

$$pK_a = pH + \log (S_T - [B]_s)/[B]_s \quad (11)$$

Thus, the pK_a can be calculated directly from the solubility data. The intrinsic solubility, $[B]_s$, of the free base was determined to be 0.12 mg/mL from experiments performed at pH 9.0. Using this value and the solubility data at pH 4.75–7.0 a pK_a value of 7.00 ± 0.03 was obtained. This value is similar to that of pilocarpic acid monoesters (6.94–6.98).[1] In Fig. 11 the curve drawn is constructed from these values and eq. 10.

The fumarate salt of **3h** is less soluble than the nitrate salt. Whereas the aqueous solubility of the latter is >8% w/v, the fumarate shows a solubility of about 1.2%.

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