This absorbed water, amounting to as much as 24 wt. % at about 90% R.H., results in reversible expansion of the lattice, especially along the *b* crystallographic direction. This, in turn, causes remarkable changes in X-ray diffraction patterns, density, and other physical properties.

A relative humidity higher than about 93% causes cromolyn sodium to absorb more water than the crystals can hold. They then collapse to form one of two lyotropic mesophases, containing from about 45 to as much as about 140 molecules of water per molecule of cromolyn sodium at 20°.

These data should be very helpful to plant processing and formulation experts. With this background, production and research personnel can be more sure that they have the best processing and storage procedures as well as the best dosage form. Problems arising in the plant or during storage and use are also more quickly solved. No important drug should be marketed without a careful study of its solid-state chemistry.

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Note added in proof: Recent X-ray work on the mesophases showed two important new results: (*a*) a single long spacing for the nematic mesophase, confirming a random arrangement of planar molecules, and (*b*) two shorter spacings as well for the middle mesophase related in the ratio $1:1/\sqrt{3}:1/\sqrt{4}$. The latter means, in turn, that the middle phase is properly named and consists of cylindrical assemblages of cromolyn sodium molecules about 25 Å in diameter and spaced from 25 to 37 Å apart, depending on water content.

Quantitative Analysis of Microgram Quantities of Pilocarpine in Aqueous Solution

A. REPTA and T. HIGUCHI

Abstract □ Aqueous solutions containing microgram quantities of pilocarpine and pilocarpic acid were assayed quantitatively, using a kinetic method based on the catalytic activity of the imidazole portion of the alkaloids on the hydrolysis of 2,4-dinitrophenyl acetate. The results were accurate to better than ±5% over a range of concentration of 6–60 mcg. of pilocarpine/ml. The effects of temperature, salt concentration, and pH were studied. Procedures for analysis of mixtures of pilocarpine and pilocarpic acid are given. Also a method is described for the analysis of small volume samples containing less than 1 mcg. of pilocarpine.

Keyphrases □ Pilocarpine and pilocarpic acid—kinetic analysis, hydrolysis of 2,4-dinitrophenyl acetate □ Pilocarpic acid and pilocarpine—kinetic analysis, hydrolysis of 2,4-dinitrophenyl acetate □ Kinetic analysis—pilocarpine and pilocarpic acid aqueous solutions

While numerous assay procedures are available for the determination of pilocarpine, including colorimetric, volumetric, polarimetric, and polarographic, (1, 2), all have some drawback. Among the drawbacks are a lack of sensitivity to small quantities of the alkaloid, the inability to distinguish between pilocarpine and its degradative products, and relatively high sensitivity to some inorganic and organic substances which may be present. The present investigation was concerned with the development of an assay procedure that would be: (*a*) sensitive to microgram quantities of pilocarpine, (*b*) relatively unresponsive to many impurities, (*c*) capable of distinguishing between pilocarpine and its primary degradative product, pilocarpic acid, and (*d*) suitable for routine analysis of pilocarpine solutions.

The kinetic approach employed arose from the recognition of the substituted imidazole portion of the pilocarpine molecule as a potential catalyst for the hydrolysis of esters such as the nitrophenyl acetates. The strong catalytic effects of imidazole and some of its derivatives were shown previously (3–8).

While the employment of the kinetics of both catalyzed and uncatalyzed reactions in quantitative analysis is well known (9–11), most of the methods used involve inorganic species and/or enzymes. The procedure described here represents one of the first successful applications of kinetic analysis to relatively simple organic molecules.

EXPERIMENTAL

Equipment and Chemicals—An Orion model 801 pH meter was used for pH determinations. Cary models 14 and 15 spectrophotometers were used for absorption measurements. Titrations were carried out using a Scientific Industries model 200 ultra-buret. Small volumes of liquid were measured using Hamilton syringes. Graduated 5-ml., glass-stoppered, tapered-tip, Pyrex centrifuge

tubes were used as reaction vessels in the assay procedure. Temperatures were controlled to ±0.1° for all studies by use of a thermostated water bath. Pilocarpine nitrate USP[1] and isopilocarpine nitrate[2] were used. The water used was distilled from acid permanganate solution in an all-glass apparatus. All other chemicals were of analytical grade. Calculations were carried out using the Olivetti Programma 101.

Methods—*A. Determination of Catalytic Effects of Pilocarpine, Pilocarpic Acid, Isopilocarpic Acid, and Isopilocarpine on Hydrolysis of 2,4-Dinitrophenyl Acetate in 5.5 ml. of Solution*—Borate buffers were prepared by addition of 0.0625 *M* sodium tetraborate solution to 0.25 *M* boric acid solution to yield the desired pH. The buffer system used in these studies consisted of 5 ml. of borate and 40 ml. boric acid stock solution. The resulting buffer solution had pH 7.45; but when the ester and sample solution were added, the final pH was 8.2 and this pH remained constant throughout the reaction.

Sample solutions containing from zero to nearly 50 mcg. of alkaloidal material/ml. of aqueous solution were prepared by dilution of a stock solution of known concentration. The preparation of the stock solutions of pilocarpic and isopilocarpic acid is discussed in Part D. The dilutions of the intact alkaloid were made just prior to their use to avoid the problem of hydrolysis which may occur in dilute solutions. The substrate solution consisted of the ester 2,4-dinitrophenyl acetate dissolved in acetonitrile, which had previously been dried over a Linde 4-A molecular sieve. The concentration was about 140 mcg. of 2,4-dinitrophenyl acetate/ml. of solution. All solutions were placed in a water bath at 25° for at least 0.5 hr. prior to use. After equilibration, 4 ml. of one of the pilocarpine solutions was pipeted into a 5-ml. glass-stoppered centrifuge tube[3], which had been temperature-equilibrated in the water bath. Then 1 ml. of buffer and 0.5 ml. of the ester solution were added. The contents were mixed and transferred to a stoppered 1-cm. quartz cell, and the absorbance changes were followed at 357 nm. The temperature of the sample compartment of the instrument was maintained at 25°.

In the early stages of this work, the increase in absorbance with time was followed continuously for more than 40% of the reaction. The sample was then allowed to stand until the reaction was complete. The limiting absorbance (A_∞) was then determined, and the observed rate constant was determined by plotting log ($A_\infty - A_t$) *versus* time in the usual manner. When the reaction appeared to have a half-life of more than 20 min., the absorbance was measured at suitable time intervals and the sample was replaced in the water bath when measurements were not being made. The absorbances and corresponding times were again plotted as described. In the terminal phases of the study and especially in checking the analytical method, only three absorbance readings were made. They were made: (*a*) immediately following addition of the ester, (*b*) at some time during the reaction, and (*c*) after the completion of the reaction. The absorbance and corresponding times were then used in the integrated first-order rate law, and the observed rate constant was calculated. It was found that all three of the described procedures for following the absorbance changes yielded results that were in good agreement.

B. Determination of Catalytic Effects of Pilocarpine in 2,4-Dinitrophenyl Acetate in 110 µl. of Solution—Those studies involving only small volumes of sample solution were carried out using the same buffer system already described. The 2,4-dinitrophenyl acetate solution used contained about 1.25 mg. of ester/ml. of acetonitrile solution. Solutions containing 0–15 mcg. of pilocarpine nitrate/ml. were prepared in 4 parts water and 1 part borate buffer. A volume of 100 µl. of this solution was placed in a centrifuge tube. To this was added 10 µl. of the ester solution, and the time of addition (t_0) was recorded. At some point during the reaction, the sample was diluted by the addition of 3 ml. of a solution containing 1 part buffer and 4 parts water. The time of dilution (t_d) was recorded. Part of the diluted sample was immediately transferred to a 5-cm. cell, and the absorbance at 357 nm. was recorded. The times at which the absorbance was first recorded (t_r) were noted, and the small change in absorbance was followed for 1–2 min. Use of this data in estimating the absorbance value (A_d) corresponding to the time of dilution (t_d) is discussed later.

The initial absorbance (A_0) was determined as follows: The ester solution was added to 3.1 ml. of the diluted buffer, and the time of addition (t_0) was recorded. The resulting solution was transferred to the 5-cm. cell, and the time at which the absorbance was first recorded (t_r) was noted. The small absorbance changes were recorded for several minutes after the initial reading. The initial absorbance (A_0) was estimated by a linear extrapolation of the recorded curve for a distance corresponding to the time that elapsed ($t_r - t_0$) from addition of the ester until the initial recorded value of the absorbance. The absorbance (A_d) corresponding to the time of dilution of the reaction mixture was estimated in a similar manner, using the elapsed time and recorded curve of each sample. The limiting absorbance (A_∞) for a series of samples was obtained by diluting each of several 110-µl. samples with 3 ml. of 0.1 *N* sodium hydroxide and measuring absorbance at 357 nm. Values obtained in this fashion agreed within ±0.5% with equilibrium measurements made on regular samples.

C. Partitioning of Pilocarpine between Chloroform and Aqueous Bicarbonate Solutions—The partition coefficient of pilocarpine between chloroform and 0.15 *M* aqueous potassium bicarbonate solution was found to be about 40. The procedure consisted of preparing solutions of known concentration of pilocarpine nitrate in water. Some exact volume (V_0) of chloroform and some exact volume (V_a) of aqueous pilocarpine nitrate solution of known concentration (C_p) were placed in a separator. A quantity of solid anhydrous potassium bicarbonate sufficient to make the aqueous volume 0.15 *M* in bicarbonate was then added to the separator. The contents were shaken vigorously and allowed to separate. The chloroform layer was drained off into a suitable stoppered centrifuge tube. To avoid the problem associated with the incomplete recovery of the chloroform from the separator, some volume (V_t) of the separated chloroform layer was pipeted into another tube. (For example, if 5 ml. of aqueous solution and 5 ml. of chloroform were used in the separator, after extraction the chloroform layer was separated and 4 ml. was pipeted into another container. V_t refers to the 4 ml. which, in this example, represented 80% of the total pilocarpine in the 5 ml. of chloroform used.) The measured volume of chloroform was then evaporated to dryness in an oil bath at about 65°. The residue was dissolved in 5 ml. of glacial acetic acid and then titrated with 0.01 *N* perchloric acid in glacial acetic acid, using 2 drops of 0.2% *p*-naphtholbenzein in glacial acetic acid as the indicator. The amount of pilocarpine, in moles, found in the titrated sample was multiplied by V_0/V_t. The value thus obtained was Q_0, the moles found in the total chloroform layer. The quantity of pilocarpine nitrate in the original aqueous solution was $V_aC_p = Q_t$ and represented the total moles of pilocarpine in the system. Thus, $Q_t - Q_0 = Q_a$, the moles of pilocarpine in the aqueous layer after extraction. The extraction coefficient can then be calculated from:

$$K_{CHCl_3/aqueous} = \frac{C_0}{C_a} = \frac{Q_0 V_a}{Q_a V_0} \qquad \text{(Eq. 1)}$$

D. Preparation of Pilocarpic Acid and Isopilocarpic Acid Solutions—Solutions of pilocarpic acid were prepared by pipeting 2 ml. of a 1% aqueous pilocarpine nitrate solution into a 100-ml. flask and adding 5 ml. of 0.1 *N* aqueous sodium hydroxide. The resulting solution was allowed to stand for 2–4 hr. to assure complete hydrolysis. Then 5 ml. of 0.1 *N* aqueous hydrochloric acid was added[4], and the solution was immediately brought to volume with water at 25°. Solutions of the desired concentration of pilocarpic acid were then prepared by dilution with water. The pilocarpic acid solutions were used on the day they were prepared. An identical procedure utilizing isopilocarpine nitrate was used for preparing isopilocarpic acid.

E. Analysis of Aqueous Solutions Containing Pilocarpine and Pilocarpic Acid—Solutions containing known concentrations of pilocarpine and pilocarpic acid were prepared by admixture of stock solutions. A portion of each of the resulting solutions was treated as described in Part A, and the observed rate constant, $k'_{obs.}$, was calculated. Another portion, consisting of 5 ml., was extracted with 5

[1] Mallinckrodt.
[2] Aldrich Chemical Co.
[3] These tubes were found to be suitable reaction vessels due to their shape, ease of handling, and the graduate markings which served as a rapid check to ensure that correct volumes of the various fluids had been added.

[4] The pH of the solution after addition of the hydrochloric acid was not allowed to fall below 4.0 in order to avoid any significant cyclization of pilocarpic acid to pilocarpine.

ml. of chloroform as described in Part D. Four milliliters of the chloroform extract was evaporated to dryness, and the residue was dissolved in 4 ml. of water. This solution was then treated as described in Part A, and the observed rate constant, $k_{obs.}$, was determined. It was approximated that extraction of pilocarpine into chloroform was complete; thus the difference between the observed rate constants was related to the concentration of pilocarpic acid, as shown in Eq. 8.

F. Temperature Dependence of Hydrolysis of 2,4-Dinitrophenyl Acetate—The observed rate constant for the hydrolysis of 2,4-dinitrophenyl acetate was carried out at 15, 25, and 35°, using the procedure described in Part A. One sample solution contained 15.4 mcg. of pilocarpine/ml. while the other contained no pilocarpine.

G. Effects of Sodium Chloride—Aqueous solutions containing 30 mcg. of pilocarpine/ml. were prepared. The 2,4-dinitrophenyl acetate solution was the same as used in Part A. Borate buffers of varying compositions were used in order that the final pH of the reaction mixture was 8.2. As the sodium chloride concentration was increased, the ratio of volumes of boric acid to borate solution was increased from 5:40 for the solution containing no sodium chloride to 12:40 for the 5% NaCl solution to 16.5:40 for the solutions containing 10% sodium chloride.

H. Determination of Catalytic Effects of Pilocarpine on Hydrolysis of 2,4-Dinitrophenyl Acetate at pH 7.9 and 8.5—The procedure used was the same as that described in Part A, except for the pH of the borate buffers used. Borate buffers prepared by mixing 40 ml. of the boric acid and 10 ml. of the borate stock solution had a pH of 7.82. After addition of the ester and sample solutions, the pH immediately rose to a value of 8.50. When 47 ml. of boric acid solution was mixed with 3 ml. of borate stock solution, the pH = 7.24; the pH of the solution obtained after addition of ester and sample was 7.90.

RESULTS AND DISCUSSION

Theoretical Considerations—The catalyzed hydrolysis of various esters by imidazole and imidazole derivatives was extensively studied (3–8). It was shown in the case of activated esters, such as the nitrophenyl acetates, that the catalysis at relatively neutral pH is due to nucleophilic attack of the free form of imidazole on the carbonyl carbon of the ester (4–6). The *N*-acyl imidazole thus produced then undergoes solvolysis to regenerate the imidazole. The present study was based on the predictions that pilocarpine, which contains a substituted imidazole, would also be an effective catalyst for the hydrolysis of certain esters, and the extent of catalysis would be a measure of the pilocarpine concentration. Based on the large body of data available for the hydrolysis of *p*-nitrophenyl acetate, it would ordinarily have been the substrate of choice in the present investigation. However, the half-life of that ester is measured in days in near neutral aqueous solution while the half-life of 2,4-dinitrophenyl acetate (DNPA) is of the order of hours under similar conditions (4). Thus, 2,4-dinitrophenyl acetate was chosen so that the assays could be done in a convenient time period. The hydrolysis of 2,4-dinitrophenyl acetate was monitored by observing the increasing absorption at 357 nm. resulting from production of the 2,4-dinitrophenoxide ion. The rate of hydrolysis of 2,4-dinitrophenyl acetate in borate-buffered solutions was found to be first order in the ester. The observed rate constant was found to be a linear function of the pilocarpine concentration, [*P*], at a given pH (Fig. 1). An acceptable rate expression for the reaction is shown in Eq. 2:

$$\frac{-d(\text{DNPA})}{dt} = (k_{H_2O} + k_{OH}[OH] + k_B[B] + k_p[P])\,[\text{DNPA}] \qquad \text{(Eq. 2)}$$

where k_{H_2O} is the pseudo-first-order rate constant for water hydrolysis, k_{OH} is the specific second-order rate constant for the hydroxide-ion-catalyzed reaction, and k_B and k_p are overall second-order rate constants for the buffer and the pilocarpine-catalyzed reaction, respectively. With any one given set of reaction conditions, such as in Fig. 1, the values of k_{H_2O}, k_{OH} [OH], and $k_B[B]$ became constant and Eq. 2 simplified to Eq. 3:

$$\frac{-d[\text{DNPA}]}{dt} = (k_0 + k_p[P])\,[\text{DNPA}] \qquad \text{(Eq. 3)}$$

where $k_0 = k_{H_2O} + k_B[B] + k_{OH}[OH]$. Thus, the observed rate constant for hydrolysis of 2,4-dinitrophenyl acetate under specified conditions may be expressed as:

$$k_{obs.} = k_0 + k_p[P] \qquad \text{(Eq. 4)}$$

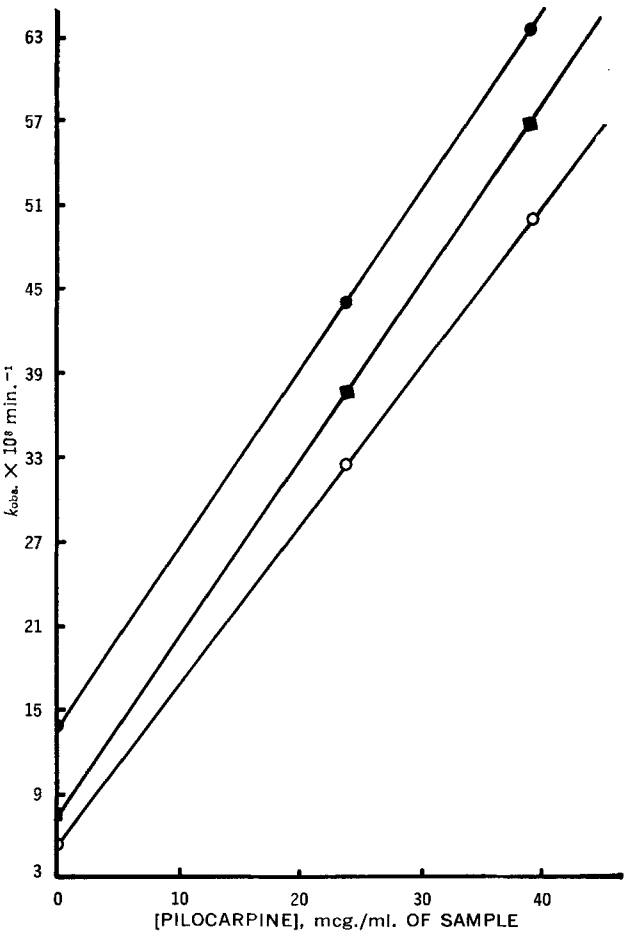

Figure 1—*Effects of pH and pilocarpine concentration on the observed rate of hydrolysis of 2,4-dinitrophenyl acetate in borate buffer solution at 25°. Key: ●, pH 8.5; ◆, pH 8.2; and ○, pH 7.9.*

The sensitivity of an assay procedure for pilocarpine based on Eq. 4 is obviously a function of the difference between k_0 and $k_{obs.}$ and the accuracy and reproducibility of their determination. For a given level of variation in reproducibility, the larger k_p is relative to k_0, the more sensitive will be the method. Thus, the ratio of k_p/k_0 should be maximized to maximize the sensitivity (10). To the end that k_0 be kept small, the buffer system chosen was borate, which apparently is a poor catalyst for ester hydrolysis. Also the buffer concentration employed was only that necessary to give suitable buffer capacity over the range of pilocarpine and ester concentrations employed.

Effects of pH—The effect of pH on the observed rate constant for 2,4-dinitrophenyl acetate hydrolysis is shown in Fig. 1. The k_p/k_0 values decrease from about 0.28 at pH 7.9 to 0.18 at pH 8.2 to 0.09 at pH 8.5. The system with pH 8.2 was chosen for further study and the development of the analytical procedure. The choice of these conditions represented a compromise between sensitivity and ease of application of the conditions to a routine analytical procedure, since at pH 8.2 the uncatalyzed reaction had a half-life of about 95 min., as compared to about 2.5 hr. at pH 7.9. In addition, the buffer capacity of the system at pH 7.9 was barely sufficient to maintain a constant pH.

Kinetic Analytical Procedure—Figure 2 shows the linearity of the dependence of $k_{obs.}$ on [*P*] at pH = 8.2. The least-squares best fit calculation yielded $k_0 = 7.2 \times 10^{-3}$ min.$^{-1}$ and $k_p = 1.28 \times 10^{-3}$ ml. mcg.$^{-1}$ min.$^{-1}$. Variations in repeated measurements were found to be less than ±5%. The experimental method used for the analytical procedure and for the determination of the rate dependence of 2,4-dinitrophenyl acetate hydrolysis on [*P*] consisted of mixing

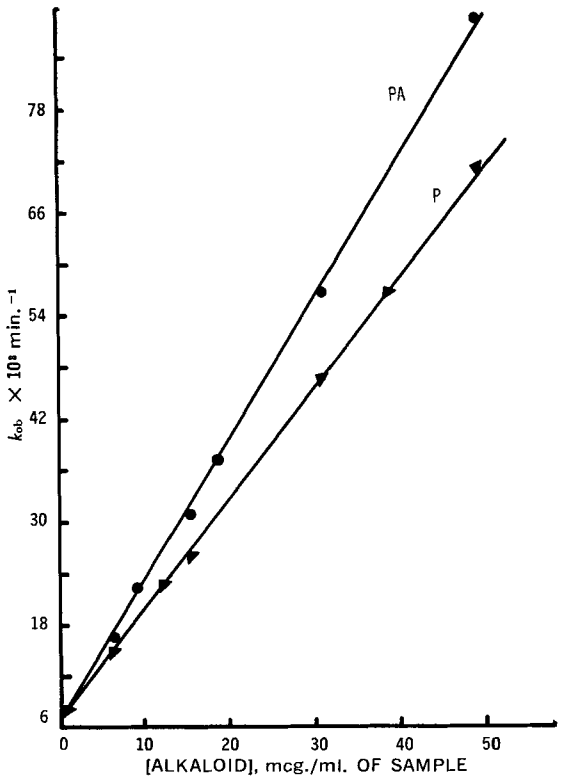

Figure 2—$k_{obs.}$ *for the hydrolysis of 2,4-dinitrophenyl acetate in borate buffer, pH 8.2, at 25° as a function of the concentration of the alkaloid pilocarpine* (P) *and pilocarpic acid* (PA) *obtained by hydrolysis of pilocarpine. Intercept* = k_0 = *7.2* × 10^{-3} *min.*$^{-1}$; *slope* (PA) = k_{PA} = *1.63* × 10^{-3} *ml./mcg.-min.; and slope* (P) = k_p = *1.28* × 10^{-3} *ml./mcg.-min.*

4 ml. of the sample solution containing pilocarpine as the nitrate salt, 1 ml. of 0.25 *M* pH 7.46 borate buffer, and 0.5 ml. of 2,4-dinitrophenyl acetate in acetonitrile. The addition of the sample and ester solution resulted in a rise in the pH to a value of 8.2, which remained constant throughout the reaction.

Several approaches were used in obtaining the absorbance–time data necessary for the calculation of the observed rate constant and [*P*]. The most convenient method consisted of making only three measurements at various times: an initial absorbance (A_0) at the beginning of the reaction (time = t_0), an absorbance (A_t) at a suitable time (t) during the reaction, and finally the limiting absorbance (A_∞) at some time (t_∞) after the reaction is complete. Using that data, $k_{obs.}$ was calculated according to Eq. 5:

$$k_{obs.} = \frac{2.303}{t - t_0} \log \left(\frac{A_\infty - A_t}{A_\infty - A_0} \right) \qquad \text{(Eq. 5)}$$

This method yielded results completely comparable to those obtained by a continuous recording of the absorbance changes. In addition, it had the advantage of greater efficiency with respect to instrument and personnel time required for each analysis.

Table I—Results of Kinetic Determination of Pilocarpine in Aqueous Solution

—Pilocarpine, mcg./ml.— Added	Found (mean)	Number of Determinations	Range of Results, mcg./ml.
46.1	46.8	2	44.4–48.4
43.0[a]	42.0	4	41.3–43.2
36.9	35.9	2	35.9–36.0
30.7[a]	30.3	11	29.3–31.7
18.4	18.3	3	18.2–18.6
12.3	12.2	2	12.1–12.3
6.1	6.4	2	6.3–6.4

[a] Values are the result of pooled data from runs on different days using different stock solutions.

Table II—Effects of Temperature on k_0, k_p, and k_p/k_0

Temperature	k_0, × 10^3 min.$^{-1}$	k_p, × 10^3 min.$^{-1}$	k_p/k_0
15°	2.7	0.77	0.28
25°	7.2	1.28	0.18
35°	19.5	1.88	0.01

Results of several analyses are shown in Table I. In all cases, the procedures used gave accuracy and precision of ±5%. All results are expressed as micrograms per milliliter of pilocarpine in the sample solution.

Effects of Temperature—The effect of temperature on the $k_{obs.}$ for the hydrolysis of 2,4-dintrophenyl acetate in the presence and absence of pilocarpine was investigated. It was expected that the pilocarpine-catalyzed rate would exhibit a lesser temperature dependence than the spontaneous rate. The observed rates of hydrolysis of 2,4-dinitrophenyl acetate was studied at 15, 25, and 35° in samples that differed only in that one series of solutions contained 15.4 mcg. of pilocarpine/ml. of sample while the other series contained no pilocarpine.

An Arrhenius plot of the data obtained showed a marked difference between the apparent activation energies of k_0 and k_p, the former being about 19.7 kcal./mole and the latter only 8.6 kcal./mole. This value of k_p compares favorably with the value of 8.4 kcal./mole obtained by Bruice and Benkovic (8) for the imidazole-catalyzed hydrolysis of *p*-nitrophenyl acetate. This observed difference in the temperature dependence of k_0 and k_p is important from the standpoint of the sensitivity of the analytical procedure. As previously discussed, the larger the ratio k_p/k_0, the greater is the sensitivity of the method. Table II contains the k_0, k_p, and k_0/k_p values obtained at the various temperatures. Inspection of this data suggests that, all other considerations being equal, a threefold increase in sensitivity can be realized by carrying out the kinetic analysis at 15° rather than 35°.

Effects of Sodium Chloride—Kinetic analyses of pilocarpine in normal saline solution were found to give significantly higher values when compared to solutions containing little or no inorganic salts. To determine the cause of these high values, two series of solutions containing 0, 5, and 10 w/v % sodium chloride were prepared. One series contained 30 mcg. of pilocarpine/ml. of sample while the other had no pilocarpine. Each of these solutions was then diluted

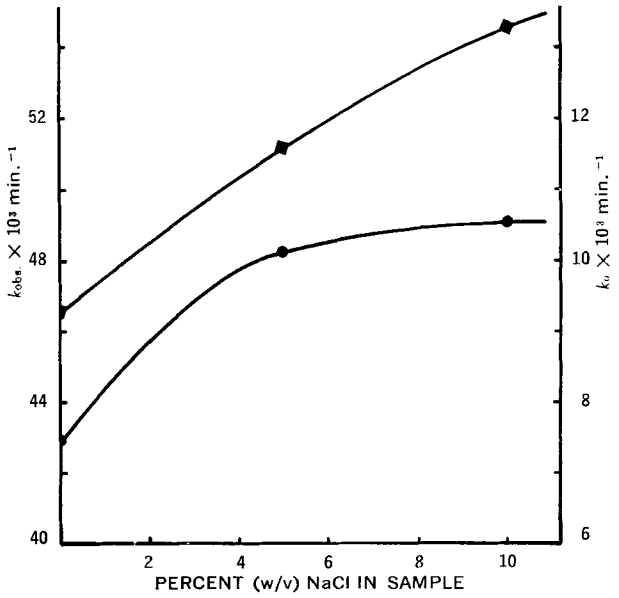

Figure 3—*Effect of sodium chloride concentration in the sample on* $k_{obs.}$ (◆) *and* k_0 (●) *at 25°. The scale on the left axis pertains to* $k_{obs.}$, *while that on the right axis pertains to* k_0.

Table III—Results of Kinetic Determination of Pilocarpine and Pilocarpic Acid in Solutions Containing Both Alkaloids

Sample[a]	Pilocarpine, mcg./ml. Added	Pilocarpine, mcg./ml. Found	Pilocarpic Acid[b], mcg./ml. Added	Pilocarpic Acid[b], mcg./ml. Found	Percent of Theoretical Found: Pilocarpine	Percent of Theoretical Found: Pilocarpic Acid
A	30.8	30.8	15.4	15.7	100.0	101.9
B	24.7	24.4	10.8	11.3	98.8	104.6

[a] Each sample was done in duplicate, and mean values were used. [b] In terms of pilocarpine hydrolyzed.

according to the normal procedure with a 0.25 *M* borate buffer of a suitable pH such that, upon dilution with the sample and addition of the ester solution, the final pH was 8.20–8.25 for all. The rate of hydrolysis of 2,4-dinitrophenyl acetate was then followed at 25° under the described conditions. Figure 3 shows that while increasing concentrations of sodium chloride increase both $k_{obs.}$ and k_0, the effects become less pronounced at higher salt concentrations. Since the relative magnitudes of the observed changes in k_0 and $k_{obs.}$ were similar, there would be no advantage realized, relative to sensitivity, by the addition of sodium chloride to the solution to be analyzed.

From these data, it can be seen that the 0.9% sodium chloride concentration of normal saline would be expected to have produced a maximum increase in $k_{obs.}$ of less than 1×10^{-3} min.$^{-1}$. The significantly larger deviation, which prompted this part of the study, could not be explained on the basis of salt catalysis. During these studies, it was noted, however, that relatively low concentrations of sodium chloride significantly increased the pH of the borate buffers; this effect was apparently responsible for the increased rates observed. These studies on salt effects did, however, point out the necessity of standardizing the reaction by use of standard solutions that are quite similar to those to be assayed.

Degradation Products of Pilocarpine—The products of the degradation of pilocarpine in aqueous solution have been suggested to be isopilocarpine (12, 13), isopilocarpic acid (12, 14, 15), and pilocarpic acid (14–16). The catalytic effects of each of these compounds on the hydrolysis of 2,4-dinitrophenyl acetate was investigated. The acids were prepared by alkaline hydrolysis of the appropriate parent alkaloids. The catalytic constants obtained for isopilocarpic acid[5], pilocarpic acid[5], and isopilocarpine were $k_{IA} = 1.59 \times 10^{-3}$ ml. mcg.$^{-1}$ min.$^{-1}$, $k_{PA} = 1.63 \times 10^{-3}$ ml. mcg.$^{-1}$ min.$^{-1}$, and $k_I = 1.27 \times 10^{-3}$ ml. mcg.$^{-1}$ min.$^{-1}$, respectively. The linear relationship between $k_{obs.}$ and $[PA]$ is shown in Fig. 2. When these values are compared with $k_p = 1.28 \times 10^{-3}$ ml. mcg.$^{-1}$ min.$^{-1}$, it is apparent that the catalytic effects of isopilocarpine and pilocarpine are the same and the catalytic effects of the acids are the same but greater than those of the intact alkaloids. The larger catalysis exhibited by the acids is believed to be due to a field effect, since they are charged species under the conditions used. Since pilocarpine and its most common degradation product catalyze the hydrolysis of 2,4-dinitrophenyl acetate, the described kinetic method may be used to assay quantitatively for any of these substances. However, it is necessary to separate the various species and, at present, there is no method by which this can be done.

The problem is simplified considerably if two conditions are assumed: (*a*) degradation of pilocarpine results in the production of pilocarpic and isopilocarpic acid only, and (*b*) the method need only distinguish between the intact pilocarpine and the therapeutically inactive acids[6]. The first assumption appears valid on the basis of recent work that showed the rate of disappearance of pilocarpine in neutral to alkaline aqueous solutions to be about the same as the rate of appearance of the acids (15, 16). The second assumption is also realistic, since only pilocarpine has been shown to have any therapeutic activity. Acceptance of these two conditions simplifies the problem to one of simply separating pilocarpine from the acids.

[5] Concentrations of the acids are calculated on the basis that 1 mcg. of the parent alkaloid is hydrolyzed to 1 mcg. of the respective acid.

[6] The significantly different rates of closure of pilocarpic acid and isopilocarpic acid to pilocarpine and isopilocarpine, respectively, were reported (15). This information may be the basis for a procedure for the analysis of mixtures of the two substances.

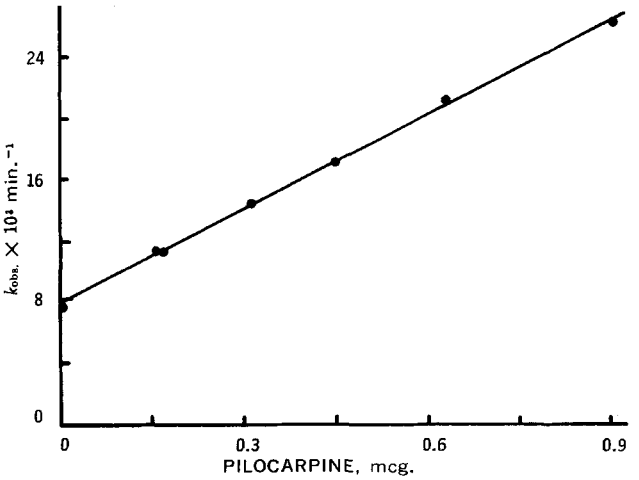

Figure 4—*Relationship between* $k_{obs.}$ *at 25° and amount of pilocarpine in 100 µl. of sample; total volume of reaction mixture is 110 µl.; buffered with borate at pH 8.2.*

Extraction Studies—Since pilocarpine is freely soluble in chloroform and pilocarpic acid and isopilocarpic acid would be in zwitterionic or anionic form at neutral to alkaline pH, and thus unlikely to be soluble in chloroform, extraction of aqueous solutions with chloroform was studied. Aqueous solutions of pilocarpine nitrate were made slightly alkaline (pH 8.5) by addition of solid anhydrous potassium bicarbonate at a concentration of 0.15 *M*. Immediately, the aqueous solution was extracted with a known volume of chloroform and the chloroform layer separated. The pilocarpine concentration in the chloroform extract was assayed both by nonaqueous titration and by the kinetic method. The results from the volumetric assays showed that 97.6% or more of the pilocarpine was extracted into chloroform when equal volumes of chloroform and aqueous solution were used. Results based on the kinetic assay indicated nearly 100% extraction under the same conditions. Based on these findings, the calculated partition coefficient[7] was $K_{o/a} \geq 40$. Similar studies on pilocarpic acid showed no detectable extraction of the pilocarpate species into the chloroform layer.

Determination of Pilocarpine and Pilocarpic Acid—Based on the results of the extraction studies, a routine was developed whereby the kinetic method could be used to determine the composition of aqueous solution containing both pilocarpine and pilocarpic acid. Solutions containing pilocarpine and pilocarpic acid were prepared by admixture of known volumes and concentrations of pilocarpine nitrate solution and pilocarpic acid solution. The observed rate constant for the hydrolysis of 2,4-dinitrophenyl acetate under normal conditions was determined. In addition, a volume of the solution containing both species was extracted with chloroform as previously discussed. A known fraction of the chloroform solution was warmed to remove all chloroform. The residue was dissolved in water, and the observed rate constant for the hydrolysis of 2,4-dinitrophenyl acetate was determined for these solutions by the kinetic procedure. From the observed rate constants and the calibrated curves in Fig. 2, the concentrations of pilocarpine and pilocarpic acid were determined according to the following equations.

For solutions containing pilocarpine (P) and pilocarpic acid (PA):

$$k'_{obs.} = k_0 + k_p[P] + k_{PA}[PA] \qquad \text{(Eq. 6)}$$

and for solutions from the chloroform extract:

$$k_{obs.} = k_0 + k_p[P] \qquad \text{(Eq. 7)}$$

$[P]$ in the solutions from the chloroform extract can be obtained directly from Fig. 2. The total pilocarpine in the original solution can then be calculated on the basis that virtually all pilocarpine is extracted. Subtracting Eq. 7 from Eq. 6 gives:

$$k'_{obs.} - k_{obs.} = k_{PA}[PA] \qquad \text{(Eq. 8)}$$

[7] $K_{o/a}$ = concentration of pilocarpine in chloroform/concentration of pilocarpine in 0.15 *M* aqueous potassium bicarbonate solution.

The left side of Eq. 8 is that part of $k'_{obs.}$ due to the catalytic hydrolysis of 2,4-dinitrophenyl acetate by pilocarpic acid; thus, $[PA]$ can be calculated from the catalytic constant for pilocarpic acid obtained from Fig. 2. Some results obtained by the method are found in Table III.

Effects of Other Nucleophiles—While no experimental attempt was made in this study to determine the effect of other nucleophiles on the rate of hydrolysis of 2,4-dinitrophenyl acetate, a substantial amount of data is available in the literature on the effects of nucleophiles on the hydrolysis of *p*-nitrophenyl acetate (8, 17). Since a free energy relationship exists between the rates of alkaline hydrolysis and imidazole-catalyzed hydrolysis of *p*-nitrophenyl and 2,4-dinitrophenyl acetate (4), the same rank and magnitude of difference in nucleophilic catalysis between imidazole and other nucleophilic substances could be expected.

In addition, since the catalytic effects of imidazole and pilocarpine are about the same, the results of Jencks and Carriuolo (17) on *p*-nitrophenyl acetate could be expected to apply in the present case. This suggests that the commonly found nucleophiles (such as carboxylate, diphosphate, and even aniline) in concentrations 10 times as great as the pilocarpine would not significantly affect the results obtained with the kinetic method. On the other hand, great care must be taken to exclude peroxides, hydrazine, hydroxylamines, and other such potential nucleophiles; even in trace concentrations, they would markedly alter the rate of the reaction and, therefore, the analytical results.

During this study, reagent grade dioxane, without further purification, was used once as a solvent for 2,4-dinitrophenyl acetate. When the solution was added to the borate-buffered sample containing no pilocarpine, the reaction went to completion in less than 3 min. This tremendous catalysis was attributed to the presence of some peroxides or peroxy acids in the dioxane, which is in qualitative agreement with published information (17).

Method for Microanalysis—If a sample containing less than 1–2 mcg. of pilocarpine is to be analyzed, the previously described method cannot be employed directly since the degree of variation in k_0 alone could exceed the catalytic effect produced by the alkaloid in the volume of solution used. However, since the method is dependent on concentration rather than amount, it should be possible to work with sufficiently small volumes of solution such that the concentration would be large enough to obtain meaningful results. For instance, if 10 ml. of solution contained 1 mcg. of pilocarpine, the normal analytical procedure could not be expected to detect this concentration. If, however, that solution is extracted with chloroform, as previously discussed, evaporated to dryness, and dissolved in 0.1 ml. of a suitable buffer system, the concentration would then be 10 mcg./ml., well within the capabilities of the kinetic procedure. Obviously, this same reasoning holds for solution volumes of less than 1 ml. having a concentration of 3–4 or more mcg. of pilocarpine/ml.

The application of the kinetic method to submicrogram quantities of pilocarpine in small volumes of solution was studied. Known amounts of pilocarpine as the nitrate salt were dissolved in a dilute buffer solution comprised of 4 parts water and 1 part 0.25 *M*, pH 7.46, borate buffer. One hundred microliters of this solution was then mixed with 10 μl. of acetonitrile solution containing 1.25 mg. of 2,4-dinitrophenyl acetate/ml. At some time, t_d, the solution was diluted with dilute buffer, and the absorbance corresponding to the time of dilution was suitably estimated. The initial absorbance (A_0) and the final absorbance (A_∞) were determined by appropriate methods, and the observed rate constants were calculated (Fig. 4). A linear dependence between $k_{obs.}$ and weight of pilocarpine was observed. Again, variation in values obtained was less than $\pm 5\%$.

On the basis of these data, it appears the procedure should be suitable for the analysis of quantities of pilocarpine as low as 100–150 ng. The intercept obtained in these studies is about 5–10% higher than that obtained with the method employing larger volumes. The value of the slopes by both methods are nearly identical when calculated in terms of the same units and concentrations. Whether or not the difference in intercepts is real or due to experimental error is not certain.

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