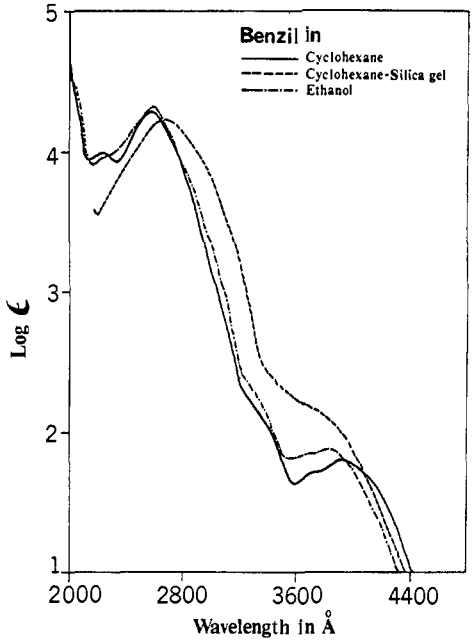

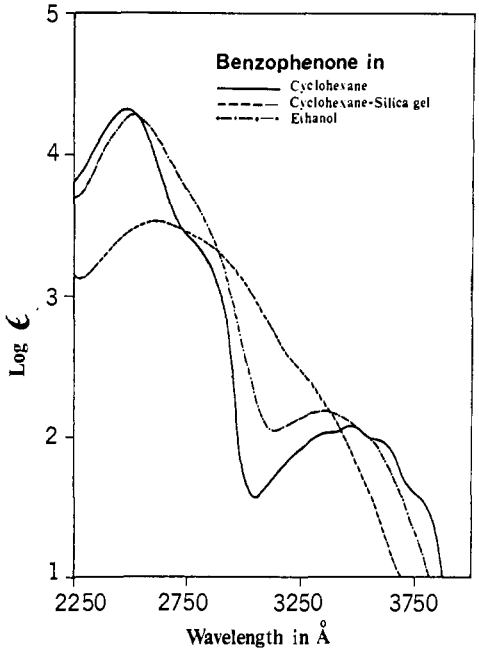

Figure 2.

135 Å. Similarly, in benzil the first $\pi-\pi^*$ band in cyclohexane is at 2570 Å. It is shifted 30 and 120 Å. in ethanol and silica gel, respectively.

The $n-\pi^*$ transitions in all of the molecules are shifted to the blue as expected. Again, the shifts are more pronounced than ethanol blue shifts. For example, the first $n-\pi^*$ transition (maximum) in tetramethyl-1,3-cyclobutanedione in cyclohexane occurs at 3510 Å. In ethanol it is shifted to the blue only 70 Å.[6] while in the silica gel–cyclohexane matrix the blue shift is 210 Å. In the next $n-\pi^*$ transition (at 3080 Å. in cyclohexane) the ethanol blue shift is only 40 Å.[6] while in silica gel the shift is 165 Å. Blue shifts in going from cyclohexane to the cyclohexane–silica gel matrix for the $n-\pi^*$ transitions of acetone and biacetyl are, respectively, 125 and approximately 325 Å. For pyruvic acid the absorption maximum in benzene at 3525 Å. becomes a shoulder about 300 Å. toward shorter wave lengths in a silica gel slurry.

This communication has been intended only to represent a few of the possibilities that the technique offers. The photochemical behavior and emission spectra of these and related systems are currently being studied.

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Titration of the Active Sites of Acetylcholinesterase[1]

Sir:

We wish to report the direct observation of a stoichiometric reaction in the eel acetylcholinesterase catalyzed hydrolysis of a carboxylic ester and the use of this stoichiometric reaction in the development of a procedure for the determination of the concentration (normality) of the active sites of this enzyme. *o*-Nitrophenyl dimethylcarbamate was used as the substrate. The initial rapid release of *o*-nitrophenol in this reaction is most readily interpreted in terms of the three-step kinetic scheme

$$E + S \underset{+\,P_1}{\overset{K_s}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' \xrightarrow{k_3} E + P_2 \qquad (1)$$

where E is enzyme, S, substrate, ES, enzyme–substrate complex, ES′, carbamyl-enzyme, P_1 and P_2 the alcohol and acid portions of the substrate, respectively, and k_2 and k_3 the rate constants of carbamylation and decarbamylation, respectively, the latter being rate-determining from the present results. The present direct observation of the two kinetic steps of eq. 1 corroborates earlier suggestions of this pathway[2] for eel acetylcholinesterase made on the basis of the reactions of phosphorus[3] and carbamate[4,5] inhibitors and noncompetitive inhibition studies.[6]

The extrapolation of the concentration of *o*-nitrophenol produced by the enzyme-catalyzed hydrolysis of this substrate to zero time gives the concentration of the active sites of the enzyme, since the following conditions are shown to be satisfied: (1) $k_2 >> k_3$[7]; (2) $S_0 >> K_m(\text{app})$[7]; (3) $S > E$[7]; and (4) the substrate reacts only at the active site. Points 2 and 3 may be experimentally controlled since all quantities are known, including $K_m(\text{app}) = 3 \times 10^{-6}$ *M*.[8] The

(6) E. M. Kosower, *J. Chem. Phys.*, **38**, 2813 (1963).

(1) This research was supported by grants from the National Institutes of Health.
(2) I. B. Wilson, F. Bergmann, and D. Nachmansohn, *J. Biol. Chem.*, **186**, 781 (1950).
(3) I. B. Wilson, S. Ginsburg, and C. Quan, *Arch. Biochem. Biophys.*, 77, 286 (1958).
(4) I. B. Wilson, M. A. Hatch, and S. Ginsburg, *J. Biol. Chem.*, **235**, 2312 (1960).
(5) I. B. Wilson and M. A. Harrison, *ibid.*, **236**, 2292 (1961).
(6) I. B. Wilson and J. Alexander, *ibid.*, **237**, 1323 (1962).
(7) L. Ouellet and J. A. Stewart, *Can. J. Chem.*, **37**, 737 (1959).
(8) J. K. Stoops, unpublished results.

quantity k_2/k_3 is found to be greater than 200.[8] This substrate reacts only at the active site since the decarbamylation rate constant determined in this titration agrees with: (1) the value determined using *o*-nitroacetanilide (the first reported amide substrate of acetylcholinesterase) to assay the enzyme-catalyzed hydrolysis of *m*-nitrophenyl dimethylcarbamate[8]; and (2) the value determined using acetylcholine to assay the decarbamylation of the dimethylcarbamylenzyme, formed from the reaction of the enzyme with dimethylcarbamyl fluoride[4,9] (see Table I).

Table I. Titration of Acetylcholinesterase Solutions with *o*-Nitrophenyl Dimethylcarbamate[a]

$S_0 \times 10^4$, *M*	$N \times 10^6$,[d] *N*	$v \times 10^9$, *M* sec.$^{-1}$	$k_3 \times 10^4$,[b,c] sec.$^{-1}$
10.03	3.7 ± 0.15[e]	2.3 ± 0.3	6.0 ± 1.0
5.016	3.7 ± 0.15	2.3 ± 0.02	6.2 ± 0.3

[a] 4.29×10^{-2} *M* Na_2HPO_4 and 4.02×10^{-3} *M* KH_2PO_4, $I = 0.2$, pH 7.72 at 25.0 ± 0.1. [b] Calculated using the expression $v = k_3N$. [c] $k_3 = 6.7 \times 10^{-4}$ sec.$^{-1}$ at 25° and pH 7.85, $I = 0.2$, using *o*-nitroacetanilide to assay the enzyme-catalyzed hydrolysis of *m*-nitrophenyl dimethylcarbamate[8]; $k_3 = 5.3$ [4] and 4.3×10^{-4} [9] sec.$^{-1}$ at pH 7.0, 25.0°. [d] V_m of phenyl acetate for this enzyme concentration was 6.14×10^{-2} *M* sec.$^{-1}$ (determined from a Lineweaver–Burk plot at pH 7.82, phosphate buffer, $I = 0.2$, 24.5°).[11] [e] The uncertainty is calculated from the uncertainty in the absorbance values of eq. 2.

The titrations reported in Table I were carried out using a Cary 14 PM recording spectrophotometer equipped with a 0.1 absorbance slide wire and a thermostated cell compartment. The enzyme was obtained from the Sigma Chemical Co., Type 3, lot 23B-7587, isolated from the electric eel and purified by the method of Lawler.[10] The enzyme solution was dialyzed against 0.2 *M* NaCl, 10^{-4} *M* phosphate at pH 7.0 at 4° for 24 hr. in order to remove ammonium sulfate. *o*-Nitrophenyl dimethylcarbamate was synthesized from the reaction of dimethylcarbamoyl chloride (Aldrich) with *o*-nitrophenol in pyridine; m.p. 56.7–57.0°. *Anal.* Calcd. for $C_9H_{10}N_2O_4$: C, 51.43; H, 4.80; N, 13.33. Found, C, 51.63; H, 4.82; N, 13.17.

A quartz cuvette with a 10-mm. light path and a capacity of 1 ml. was filled with 500 μl. of phosphate buffer, pH 7.82, and 200 μl. of enzyme solution. After determining a base line, 25 μl. of the buffer was added to the solution resulting in a small decrease in absorbance; then 25 μl. of *o*-nitrophenyl dimethylcarbamate solution in 50% (v./v.) acetonitrile–water was added and the solution was stirred for *ca.* 30 sec. The liberation of *o*-nitrophenoxide ion at 415.0 mμ was recorded ($\epsilon_{415.0}$ 4.00×10^3, measured under the reaction conditions by adding 25 μl. of *o*-nitrophenol in water to the completed reaction using the 1.0 absorbance slide wire). An instantaneous increase in absorbance of about 0.015 unit occurred followed by a slow zero-order increase in absorbance, which was recorded for 1.3 hr. The pH of the solution, determined immediately after completion of the reaction, was 7.72, the change being caused by the buffering action of the enzyme.

Using the above procedure, the concentration (normality of the active sites in the solution) is[12]

$$N = \frac{-F(A_1 - A_2) + A_3 - A_4}{4.00 \times 10^3} \qquad (2)$$

where A_1 is the initial absorbance of the enzyme solution, A_2, the absorbance after the addition of 25 μl. of buffer (which measures the absorbance change due to the dilution of the enzyme solution), A_3, the extrapolated absorbance at zero time, and A_4, the absorbance of the carbamate (measured separately). The difference of A_1 and A_2 is corrected for dilution by the factor F.

Table I shows excellent agreement between two titrations of acetylcholinesterase carried out with different substrate concentrations. The accuracy of the titration is inversely proportional to the enzyme concentration. The lower limit of the titration is about 1.5×10^{-6} N,[11] at which concentration the uncertainty is about ±10% depending on the molar absorptivity of the *o*-nitrophenoxide ion.[14] The titration itself requires less than 2 min. since the steady-state reaction is reached in less than 30 sec. for the substrate concentrations used here. Thus the normality of acetylcholinesterase active sites may be determined in an accurate and convenient manner.[15]

(9) I. B. Wilson, M. A. Harrison, and S. Ginsburg, *J. Biol. Chem.*, **236**, 1498 (1961).

(10) H. C. Lawler, *ibid.*, **234**, 799 (1959).

(11) The relation between the titration presented here and a rate assay to determine the concentration of acetylcholinesterase, using phenyl acetate as a substrate, will be presented in the full report of this work.

(12) This equation follows that for a method B titration of α-chymotrypsin with N-*trans*-cinnamoylimidazole.[13] It should be noted that a dilution factor was mistakenly omitted in that previous equation.

(13) G. R. Schonbaum, B. Zerner, and M. L. Bender, *J. Biol. Chem.*, **236**, 2930 (1961).

(14) Results consistent with those of Table I were obtained when the titration was determined at pH 8.20 at an enzyme concentration of 1.49×10^{-6} *N*.

(15) Previous titrations of acetylcholinesterase solutions have involved use of radioactive diisopropylphosphorofluoridate[16] and dimethylcarbamyl fluoride using an indirect procedure.[5]

(16) H. Michel and S. Krop, *J. Biol. Chem.*, **190**, 119 (1951).

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Rearrangement of Quadricyclanone. Genesis of the *cis*-Bicyclo[3.2.0]heptadiene System

Sir:

As reported earlier,[1] oxidation of quadricyclanol (**1**) with *t*-butyl hypochlorite and pyridine yielded quadricyclanone (**2**). We now find that oxidation of **1** under Oppenauer conditions[2] generates, instead of quadricyclanone (**2**), *cis*-bicyclo[3.2.0]hepta-3,6-dien-2-one (**3**) in about 8% yield (isolated). The only other isolable product of this oxidation was an, as yet, unidentified ketone (**4**, 1.7%) whose empirical formula was determined as $C_7H_6O_2$.

We further observed that if the oxidation conditions[2] were modified so that the reaction was conducted in benzene at room temperature and for shorter periods,

(1) P. R. Story and S. R. Fahrenholtz, *J. Am. Chem. Soc.*, **86**, 1270 (1964).

(2) For an example of the procedure followed see R. K. Bly and R. S. Bly, *J. Org. Chem.*, **28**, 3165 (1963).