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A SENSITIVE FLUOROMETRIC PROCEDURE FOR THE DETERMINATION OF SMALL QUANTITIES OF ACETYLCHOLINESTERASE*

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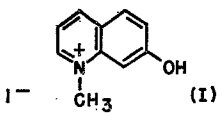
Abstract—Formation of the highly fluorescent 1-methyl-7-hydroxyquinolinium iodide during the acetylcholinesterase-catalyzed hydrolysis of 1-methyl-7-acetoxyquinolinium iodide has been utilized in the development of a simple fluorometric assay for extremely small quantities of the enzyme. By employing the methods described, quantities of enzyme as low as 0·0003 unit‡ may be determined.

IN A recent publication,[1] a spectrophotometric study of the acetylcholinesterase-catalyzed hydrolysis of a series of isomeric 1-methyl-acetoxyquinolinium iodides was described. Kinetic experiments were based on the estimation of the colored product formed during the enzyme-catalyzed hydrolysis. It was found that the smallest amount of enzyme that could be estimated by this procedure varied from 0·005 to 0·02 unit acetylcholinesterase/ml reaction mixture, dependent on the particular isomer employed. Since 1-methyl-7-hydroxyquinolinium iodide (I) was



(I)

noticeably fluorescent in solution, and since it is generally possible to estimate fluorogenic compounds at considerably lower concentrations than chromogenic ones, it appeared that a method for increasing the sensitivity of the assay was at hand.

The present study discusses the development of a simple fluorometric assay for extremely small quantities of acetylcholinesterase, based on the hydrolysis of 1-methyl-7-acetoxyquinolinium iodide to the highly fluorescent 1-methyl-7-hydroxyquinolinium iodide.

While this work was in preparation, the use of indoxyl acetate and resorufin esters as fluorogenic substrates for esterases was reported.[2] Acetylcholinesterase was shown to have little effect on resorufin esters. It did hydrolyze indoxyl acetate, but the fluorescent hydrolysis product was susceptible to oxidation, giving a nonfluorescent

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‡ 1 unit acetylcholinesterase is defined as that amount of enzyme that will catalyze the hydrolysis of 1 μmole acetylcholine/min.

product (indigo blue). This necessitated the addition of an antioxidant to the reaction mixture. This present study showed 1-methyl-7-hydroxyquinolinium iodide to be intensely fluorescent, fluorescence emission being well separated from the excitation wavelength, stable at all pH values, and thus a highly suitable material on which to base a fluorometric assay procedure.

EXPERIMENTAL

The acetylcholinesterase (from the electric organ of *Electrophorus electricus*) employed in this study had a specific activity of 46·2 μmoles acetylcholine hydrolyzed/min per mg protein (46·2 units/mg protein) as determined under standard assay conditions.[3] Protein concentration was 0·13 mg/ml in 0·03 M sodium phosphate buffer, pH 7·0.

The preparation, purification, and the visible and u.v. spectral characteristics of the hydroxyquinolinium iodides and their acetyl derivatives have been described previously.[1] An Aminco-Bowman spectrophotofluorometer equipped with a Xenon lamp, an optical unit for proper control of the fluorescence excitation and emission wavelengths, and a photomultiplier microphotometer equipped with a 1P21-type photomultiplier tube was used in all measurements. The calibration of the instrument was checked by comparison of the excitation and fluorescence spectra obtained for solutions of proflavine with the corrected data previously reported.[4] The instrument was found to be reliable in the wavelength region important for this study (see Results).

Fluorescence measurements. Instrument parameters such as cell and photomultiplier slit dimensions, as well as photomultiplier microphotometer settings, were varied to give the sensitivity and resolution demanded by each experiment. Once fluorescence had been identified and characterized, instrument parameters were changed to give the increased sensitivity required for determination of the fluorescence of low concentrations of the particular fluorescent compound under investigation. The excitation and fluorescence spectra were recorded as plots of relative fluorescence intensity (microphotometer reading in per cent transmission multiplied by multiplier switch setting $\times 10^3$) vs. wavelength of excitation or emission. Maximal sensitivity used in every case was that which gave a relative fluorescence intensity reading of 3 or less for a solvent blank on irradiation at the excitation wavelength of the particular fluorescent compound under investigation. The quantitative measurements reported in this work were obtained by use of constant slit dimensions throughout. These dimensions (in inches) were as follows: $\frac{3}{16}$, $\frac{1}{8}$, $\frac{3}{16}$, $\frac{3}{16}$, $\frac{1}{8}$, $\frac{3}{16}$, $\frac{1}{16}$, respectively, for slits 1 to 7 (slit numbers as defined in the Aminco-Bowman manual No. 768A).

Enzyme activity measurements. Rate measurements were performed at room temperature (25°), in 1-cm quartz cuvettes supplied with the instrument. Assays were carried out by addition of enzyme to a solution (2 ml) of $1{\cdot}43 \times 10^{-7}$ M 1-methyl-7-acetoxyquinolinium iodide in 0·05 M sodium phosphate buffer, pH 7·0. The solution was well mixed and measurements of the relative fluorescence intensity of the resulting solution (at the wavelength of maximal fluorescence) started 1 min later. Readings were taken every minute for several minutes and the results recorded as relative fluorescence intensity vs. time plots. In every case the resultant plots were straight lines, and therefore no difficulty was experienced in obtaining the velocity of hydrolysis

with the aid of calibrations of concentration of 1-methyl-7-hydroxyquinolinium iodide vs. relative fluorescence intensity under the same conditions. In order to determine the minimal amount of enzyme that would give a measurable rate of hydrolysis by this procedure, the stock enzyme solution was diluted as required with 0·01 M sodium phosphate buffer containing 0·05% gelatin, pH 7·0. Each series of experiments was accompanied by a blank, containing all components except the enzyme, from which corrections for the spontaneous hydrolysis of the substrate could be made. The rate of spontaneous hydrolysis of 1-methyl-7-acetoxyquinolinium iodide is directly proportional to its concentration at pH 7·0, as was previously demonstrated.[1] In order to minimize this hydrolysis, the substrate concentration was reduced as far as possible, while sufficient concentration was maintained to ensure reliable kinetics. At the substrate concentration used, the rate of spontaneous hydrolysis was $4{\cdot}08 \times 10^{-10}$ mole/liter per min.

RESULTS

Fluorescence characteristics of the 1-methyl-quinolinium iodides. Since the enzyme assays were to be carried out at pH 7·0 in 0·05 M sodium phosphate buffer, the quinolinium compounds were examined for fluorescence under the same conditions. It was found that 1-methyl-7-hydroxyquinolinium iodide fluoresces maximally at 505 mμ, with corresponding excitation bands at 260 mμ and 406 mμ. Excitation at 406 mμ gave fluorescence of greater intensity than excitation at the shorter wavelength. In Fig. 1 are shown the excitation and fluorescence spectra of 1-methyl-7-hydroxyquinolinium iodide (curves *a* and *b*). The acetyl derivative did not fluoresce at 505 mμ (on excitation at 406 mμ) but maximal fluorescence was obtained at 410 mμ with corresponding excitation bands at 240 mμ and 320 mμ (Fig. 1, curves *c* and *d*). It was observed that the fluorescence intensity of the acetyl derivative at 410 mμ (excitation 320 mμ) decreased slowly on standing, whereas the fluorescence intensity at 505 mμ (excitation 406 mμ) increased, until the final fluorescence emission and the corresponding excitation spectrum were identical with that of 1-methyl-7-hydroxyquinolinium iodide. This change was doubtless caused by spontaneous hydrolysis of the substrate. Identical, albeit accelerated, changes were noted in the presence of acetylcholinesterase.

It was further shown that the fluorescence intensity of 1-methyl-7-hydroxyquinolinium iodide at 505 mμ (excitation 406 mμ) decreased with decrease of pH, in a manner analogous to the previously reported[1] dependence of the absorption at 406 mμ on pH. The fluorescence intensity of suitable concentrations of 1-methyl-7-hydroxyquinolinium iodide was measured in 0·05 M sodium phosphate buffer, from pH 5·7 to 8·0 and also in 0·05, 0·005 N H_2SO_4 and in 0·05, 0·005 N NaOH. A plot of relative fluorescence intensity of these solutions (measured at the highest usable instrument sensitivity) vs. pH yielded a curve (Fig. 2*A*) from which the apparent pK_a of the phenolic hydroxyl group was found to be 5·8—in good agreement with the value similarly obtained by the spectrophotometric procedure.[1]

In dilute sulfuric acid the compound showed no fluorescence emission at 505 mμ on excitation at 406 mμ, but was found to fluoresce maximally at 505 mμ on excitation at 240 mμ and 350 mμ. It appears then, that both the undissociated phenol and the phenolate ion fluoresce at the same wavelength, but differ in the wavelength of the excitation required to produce the fluorescence. The maximal excitation wavelengths

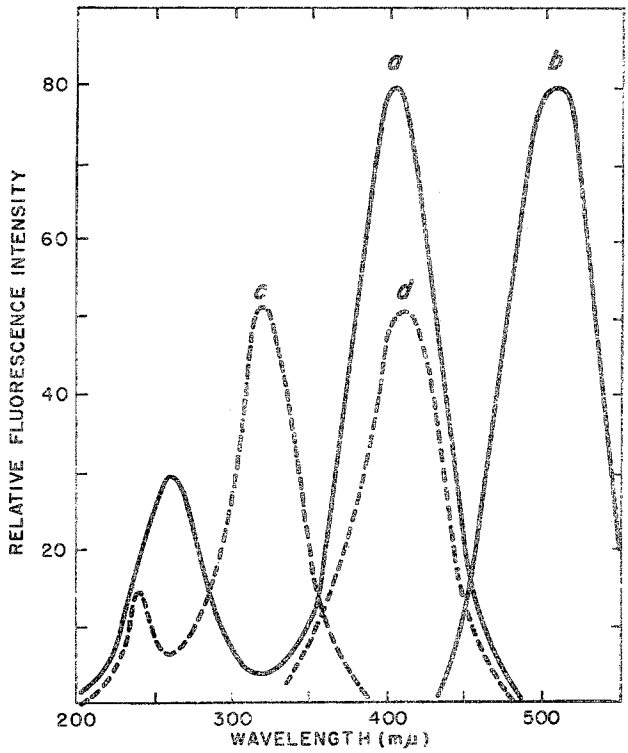

FIG. 1. The excitation and fluorescence spectra of 1-methyl-7-hydroxyquinolinium iodide and its acetyl derivative in 0·05 M sodium phosphate buffer, pH 7·0. Instrument parameters were kept constant throughout. Curve *a*: excitation spectrum of 1-methyl-7-hydroxyquinolinium iodide ($2{\cdot}4 \times 10^{-8}$ M), fluorescence measured at 505 mμ. Curve *b*: corresponding fluorescence spectrum of 1-methyl-7-hydroxyquinolinium iodide ($2{\cdot}4 \times 10^{-8}$ M), excitation wavelength 406 mμ. Curve *c*: excitation spectrum of 1-methyl-7-acetoxyquinolinium iodide (3×10^{-8} M), fluorescence measured at 410 mμ. Curve *d*: corresponding fluorescence spectrum of 1-methyl-7-acetoxyquinolinium iodide (3×10^{-8} M), excitation wavelength 320 mμ.

correspond to the highest absorption wavelengths found for the phenol and phenolate ion.[1]

Relative fluorescence intensity at 505 mμ (excitation 406 mμ) in 0·05 M sodium phosphate buffer, pH 7·0, was found to be directly proportional to the concentration of 1-methyl-7-hydroxyquinolinium iodide at all concentrations up to $1{\cdot}4 \times 10^{-7}$ M (the highest concentration studied). Figure 2*B* shows this relationship in the lowest detectable concentration range. The minimal detectable concentration of 1-methyl-7-hydroxyquinolinium iodide was found to be 3 to 5×10^{-9} M. From these calibrations, direct conversion of relative fluorescence intensity to molar concentration of the hydrolysis product could be effected.

With the same slit widths as employed in the above calibration, a 2×10^{-6} M solution of proflavine in 0·1 M acetate buffer, pH 5·5, exhibited maximal fluorescence at 510 mμ. The corresponding excitation maximum was at 444 mμ. These wavelengths are in good agreement with previously reported calibrations of proflavine under identical conditions.[4] A 4×10^{-9} M solution of proflavine in 0·1 M acetate buffer, pH 5·5, gave a relative fluorescence intensity of 38.

Of the other quinolinium compounds in this series, it was found that the 8- and 5-derivatives exhibited no detectable fluorescence. Both 6-hydroxy- and 6-acetoxyquinolinium iodide were found to fluoresce. However, unlike the 7-derivatives, both the hydroxy compound and its acetyl derivative gave fluorescence emission at the same wavelength. Excitation at 230 mμ and 310 mμ produced fluorescence at

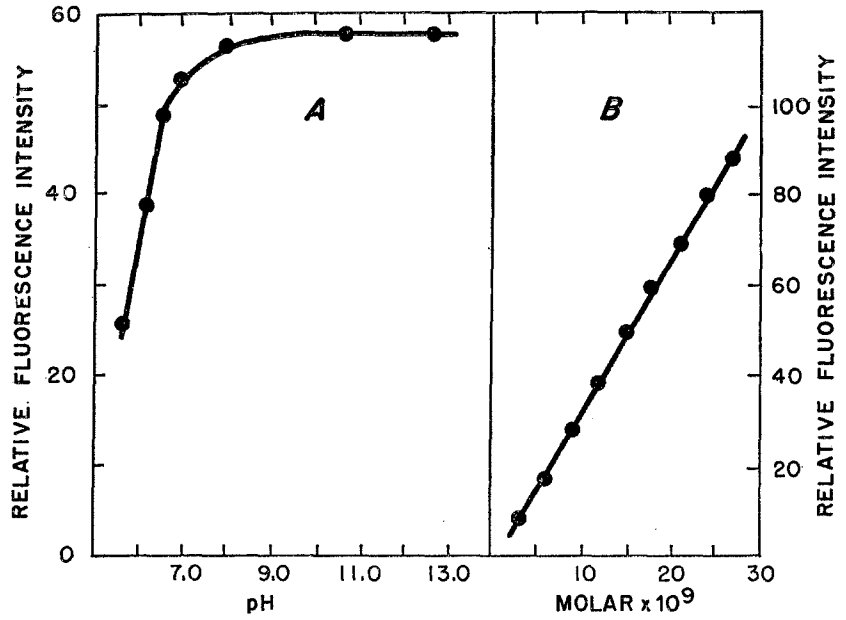

FIG. 2. *A*. Variation of fluorescence intensity at 505 mμ (excitation 406 mμ) of 1-methyl-7-hydroxyquinolinium iodide ($1{\cdot}6 \times 10^{-8}$ M) as a function of pH. Instrument parameters constant at the greatest permissible sensitivity.

B. Fluorescence intensity at 505 mμ (excitation 406 mμ) of 1-methyl-7-hydroxyquinolinium iodide as a function of concentration, in 0·05 M sodium phosphate buffer, pH 7·0. Instrument parameters constant throughout at the greatest permissible sensitivity.

410 mμ. No differences could be found in the excitation spectra of the two derivatives. As the fluorescence of both derivatives was also similar in intensity, hydrolysis of the acetyl derivative could not be observed by this procedure.

Acetylcholinesterase-catalyzed hydrolysis of 1-methyl-7-acetoxyquinolinium iodide. In Fig. 3 is shown the effect of increasing the enzyme concentration in the assay mixture, on the rate of hydrolysis of 1-methyl-7-acetoxyquinolinium iodide. Enzyme concentration was increased from the minimal concentration of 0·0003 unit/ml reaction mixture, the lowest enzyme concentration at which the enzyme-catalyzed hydrolysis of 1-methyl-7-acetoxyquinolinium iodide could be reliably measured. It is notable that over the concentration range of enzyme used, the observed rate of hydrolysis was directly proportional to enzyme concentration. It was previously reported that the activity–enzyme concentration curves at low enzyme concentrations were not linear when determined at substrate concentrations suitable for the spectrophotometric procedure.[1]

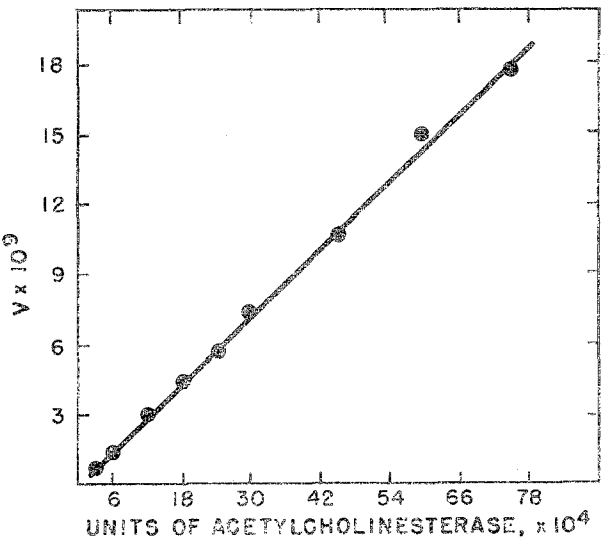

FIG. 3. Rate of hydrolysis of 1-methyl-7-acetoxyquinolinium iodide ($1{\cdot}43 \times 10^{-7}$ M) as a function of acetylcholinesterase concentration in 0·05 M sodium phosphate buffer, pH 7·0. Ordinate: rate of hydrolysis (v) in moles per liter per minute. Abscissa: concentration of acetylcholinesterase in units per milliliter reaction mixture. Each point represents the mean of four separate determinations. Fluorescence observed at 505 mμ on excitation at 406 mμ. Instrument parameters constant throughout at the greatest permissible sensitivity.

DISCUSSION

It has been shown that the fluorescence of 1-methyl-7-hydroxyquinolinium iodide and the nonfluorescence of its acetyl derivative at 505 mμ, on excitation at 406 mμ, may be conveniently used for the assay of small quantities of acetylcholinesterase. The method is based on the enzyme-catalyzed hydrolysis of the synthetic substrate, the kinetics of which have been more fully discussed elsewhere in a study of the hydrolysis of a series of acetoxyquinolinium iodides.[1]

By utilizing the fluorescence technique, the sensitivity of the assay can be increased some 20- to 30-fold over the spectrophotometric procedure. Thus, enzyme concentrations as low as 0·0003 unit/ml reaction mixture could be determined. It appears possible that this procedure could be readily adapted to the determination of small quantities of enzyme in muscle or nerve fibers, replacing the more laborious and less accurate procedures currently in use.

A further advantage offered by the procedure of measuring the amount of hydrolysis product by its fluorescence is that the activity–enzyme concentration curve is linear. The nonlinearity observed previously in the spectrophotometric procedure was ascribed to inhibition of the enzyme by the hydrolysis product.[1] In the case of the fluorometric assay, the low concentrations used would lead to the formation of only minute amounts of hydrolysis product ($\sim$1 to 3×10^{-8} M). This concentration would presumably be lower than that required to cause significant inhibition of the enzyme.

As was demonstrated in a previous paper,[1] 1-methyl-7-acetoxyquinolinium iodide is susceptible to hydrolysis by other hydrolytic enzymes, although generally at a

much lower rate than by acetylcholinesterase. Therefore, no claims regarding the specificity of the assay procedure can be made.

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