Molecular Crystals and Liquid Crystals Incorporating Nonlinear Optics
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/gmcl17

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Version of record first published: 28 Mar 2007.

To cite this article: Paul D. Hale & R. Mark Wightman (1988): Enzyme-Modified Tetrathiafulvalene Tetracyanoquinodimethane Microelectrodes: Direct Amperometric Detection of Acetylcholine and Choline, Molecular Crystals and Liquid Crystals Incorporating Nonlinear Optics, 160:1, 269-279

To link to this article: http://dx.doi.org/10.1080/15421408808083022

Mol. Cryst. Liq. Cryst., 1988, Vol. 160, pp. 269–279
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Printed in the United States of America

Enzyme-Modified Tetrathiafulvalene Tetracyanoquinodimethane Microelectrodes: Direct Amperometric Detection of Acetylcholine and Choline

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Microelectrodes constructed with the one-dimensional organic conducting salt TTF-TCNQ as the electrode material and modified with two enzymes, acetylcholinesterase and choline oxidase, are used to selectively detect the neurochemically important molecule acetylcholine and its metabolite choline. Kinetic analysis of the variation of current with substrate concentration indicates that the rate-limiting step in these electrodes is the enzyme kinetics. Values for the electrochemical rate constant are reported and the conditions of enzyme saturation are defined.

INTRODUCTION

Recent work by Albery and co-workers[1–3] has shown that a variety of one-dimensional conducting organic salts can be used as electrode materials for the direct oxidation of enzymes containing the flavin prosthetic group flavin adenine dinucleotide (FAD). For example, electrodes constructed of the salts of tetracyanoquinodimethane (TCNQ) and tetrathiafulvalene (TTF), quinoline, or N-methylphenazine were shown to efficiently oxidize glucose oxidase. Among these materials, TTF-TCNQ was found to be the most suitable due to its low background current, and enzyme-modified TTF-TCNQ electrodes have been used for the determination of biological purines,[4] as well as for the *in vivo* measurement of glucose in the rat brain.[5] In these electrodes, the FAD group in the enzyme is reduced by the

substrate and this reduced enzyme is then directly oxidized at the electrode, as shown in the reaction scheme below:[2]

$$SH_2 + E(FAD) \rightarrow S + E(FADH_2) \quad (1)$$

$$E(FADH_2) \xrightarrow{\text{TTF-TCNQ}} E(FAD) + 2H^+ + 2e^- \quad (2)$$

Here SH_2 is the reduced form of the substrate and E(FAD) represents the oxidized form of the corresponding flavoenzyme.

In this paper we report the design and response of enzyme-modified TTF-TCNQ microelectrodes for the determination of the neurotransmitter acetylcholine (ACh), $CH_3CO_2CH_2CH_2N^+(CH_3)_3$, and its metabolite choline (Ch), $HOCH_2CH_2N^+(CH_3)_3$. The role of ACh as a neurotransmitter has been known for many years, and is well documented.[6] Several methods for the determination of this species have recently been developed, including radio-labelling[7,8] and gas chromatographic[9] techniques. In addition, experiments utilizing high-performance liquid chromatography (HPLC) with electrochemical detection have been reported.[10,11] In this latter work, the actual detected species is hydrogen peroxide, which is the electroactive by-product of the following enzymatic reactions:

$$\text{acetylcholine} + H_2O \xrightarrow{\text{AChE}} \text{choline} + \text{acetic acid} \quad (3)$$

$$\text{choline} + 2O_2 + H_2O \xrightarrow{\text{ChO}} \text{betaine} + 2H_2O_2 \quad (4)$$

In this scheme, AChE and ChO refer to acetylcholinesterase and choline oxidase, respectively. Although the HPLC-electrochemical systems are sensitive and specific in the measurement of ACh and Ch, the response is strongly affected by the ambient concentration of oxygen, as indicated in equation 4 above. More importantly, the time response of these systems is quite long, which precludes their use as *in vivo* probes.

In the present work, the use of TTF-TCNQ microelectrodes modified with acetylcholinesterase and choline oxidase permits a more direct measurement of both Ch and ACh, with improved time response over the systems described above and excellent spatial characteristics. For the detection of Ch, these electrodes follow the scheme outlined in (1) and (2); in the case of ACh, the substrate is first converted to Ch according to (3). The processes occurring at the

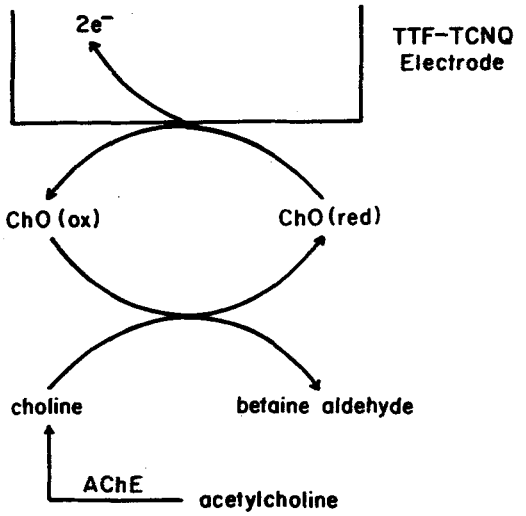

FIGURE 1 Schematic representation of the double-enzyme TTF-TCNQ electrode. AChE refers to acetylcholinesterase; ChO(ox) and ChO(red) refer to the oxidized and reduced forms of choline oxidase, respectively.

electrode surface are shown schematically in Figure 1. In the following sections, the response of these electrodes to ACh and Ch is described in more detail, and the results are analyzed using the theory for amperometric enzyme electrodes developed by Albery and Bartlett.[1]

EXPERIMENTAL

Reagents. Acetylcholinesterase from electric eel (EC 3.1.1.7; Type III, activity 970 units/mg) and choline oxidase from Alcaligenes species (EC 1.1.3.17; activity 13.9 units/mg) were obtained from Sigma. The TTF and TCNQ were obtained from Aldrich. All other chemicals were reagent grade and were used as received. Doubly distilled water was used in the preparation of all solutions. All experiments were carried out in phosphate buffer containing NaCl (0.1*M*), which was prepared by titrating 0.1*M* Na_2HPO_4 to a pH of 7.4 with concentrated HCl. Except when testing for oxygen interference, all experimental solutions were deaerated by bubbling N_2 through the solution for at least 10 min.

Electrode Preparation. The TTF-TCNQ complex was prepared using a standard procedure.[12] A slurry of the conducting salt and

poly(vinyl chloride) (PVC) in tetrahydrofuran (in a 10:1 ratio of TTF-TCNQ:PVC by weight) was used to partially fill a glass capillary (1.3 mm outer diameter, 0.75 mm inner diameter, A-M Systems, Inc., Everett, WA). After evaporation of the solvent, a solid plug of TTF-TCNQ/PVC remained in the bottom of the capillary; the plug was typically 3 to 5 mm in length, with a diameter of approximately 0.6 mm as measured by electron microscopy. The electrode material was then sealed in the capillary with epoxy (Epon 828 with 14% metaphenylenediamine, Miller Stephenson Chemical Co., Danbury, CT) and cured for 1 hour at 80C. Afterwards, the electrode surface was polished with fine emory paper. Electrical contact was established by injecting mercury into the top of the capillary and inserting a silver wire.

The immobilized enzyme electrode was prepared by soaking the TTF-TCNQ electrode in an aqueous solution of acetylcholinesterase and choline oxidase for several hours. Unlike most of the previously published work,[1–4,13] a dialysis membrane was not used to trap the enzyme solution; work in this laboratory and elsewhere[5] indicates that the enzymes remain incorporated in the electrode for long periods without a membrane.

Apparatus. The constant potential experiments were run using a Princeton Applied Research Polarographic Analyzer (Model 174A) and a Houston Instruments strip chart recorder. In addition to the TTF-TCNQ working electrode, a saturated sodium calomel reference electrode (SSCE) and a platinum wire auxiliary electrode were employed. All current measurements were made at a constant potential of +150 mV vs. the SSCE, and the experiments were conducted at 23(±2)C.

RESULTS AND DISCUSSION

Figure 2 shows typical results for the response of the enzyme-modified TTF-TCNQ microelectrode to ACh in pH 7.4 phosphate buffer. Steady state values for the current are achieved quite rapidly, with a delay of less than 1 min. from the addition of substrate to the steady state measurement. The electrodes appear to regenerate the oxidized form of choline oxidase very efficiently. In several cases, the test solution was not deaerated in order to test for oxygen interference (i.e. to see if the enzyme would be oxidized by O_2 rather than at the electrode); no decrease in current was apparent. For the majority of electrodes tested, the maximum current was approximately the same for both

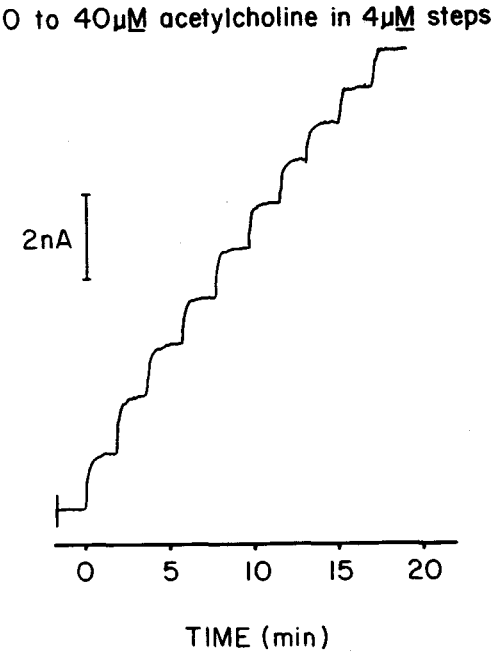

FIGURE 2 Typical results for the variation of current with acetylcholine concentration for the acetylcholinesterase/choline oxidase TTF-TCNQ electrode.

Ch and ACh, as the calibration curves in Figure 3 indicate, but enzyme saturation was usually reached at lower concentrations when Ch was used as the substrate; this point will be discussed in more detail below. In order to obtain kinetic information, these curves are analyzed using a theory developed by Albery and co-workers.[1-3] As a first step, it is necessary to construct Hanes plots[14] of $[S]/i$ versus $[S]$, where $[S]$ is the substrate concentration and i is the measured current. From these plots, one can find a limiting value of the electrochemical rate constant k'_{ME} for low substrate concentrations, as described by the equation below:

$$k'_{ME} = \{i/nAF[S]\}_{[S]\to 0} \tag{5}$$

Here A is the surface area of the electrode, n is the number of electrons transferred, and F is the Faraday constant. The rate constant is thus determined by extrapolating the Hanes plot to zero concentration. The Hanes plots for the data in Figure 3 are presented in Figure 4, and the electrochemical rate constants resulting from a large

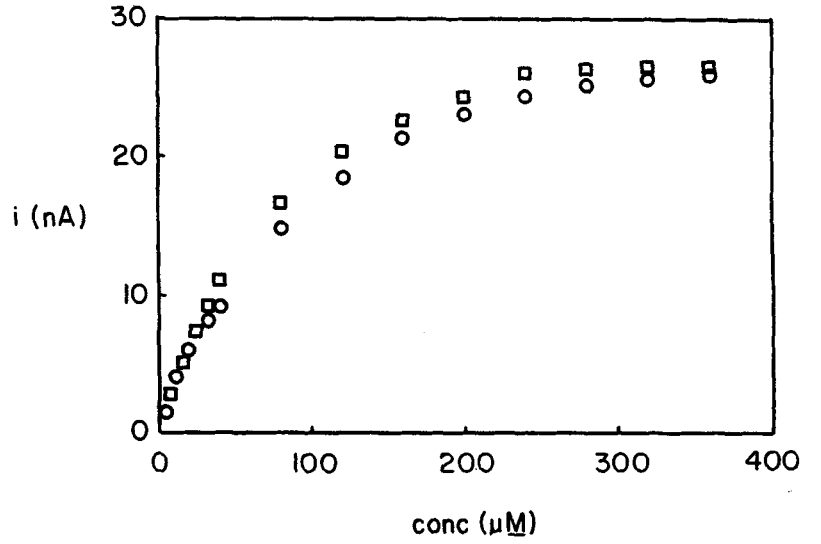

FIGURE 3 Calibration curves for the variation of current with substrate concentration for the acetylcholinesterase/choline oxidase TTF-TCNQ electrode: (□) choline; (○) acetylcholine.

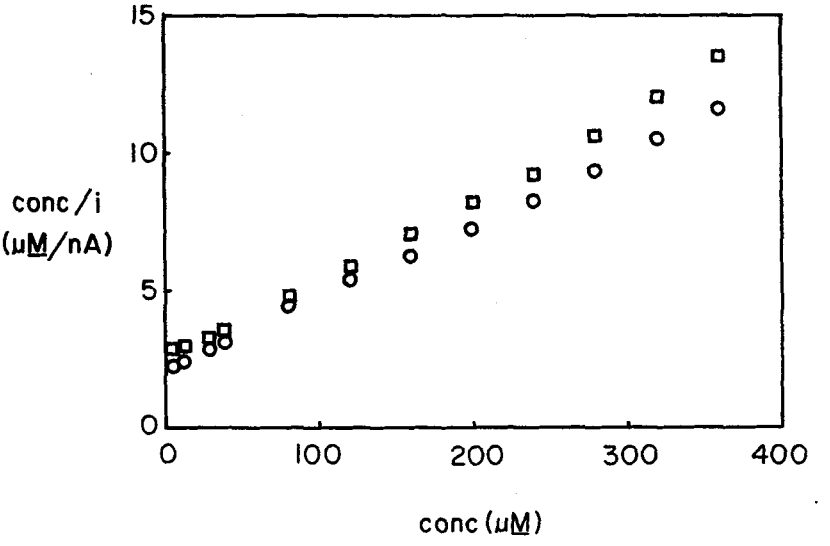

FIGURE 4 Hanes plots[1,14] for the data shown in Figure 3: (□) choline; (○) acetylcholine.

number of measurements are listed in Table I. It should be noted that one would expect that the k'_{ME} values found for the detection of Ch and ACh by the two-enzyme system should be nearly equal since acetylcholinesterase is one of the most efficient enzymes known[6]; i.e. the conversion of ACh to Ch (equation 3) is sufficiently rapid that this step can be ignored in the kinetic treatment. The experimental results support this point, giving nearly the same electrochemical rate constant for both Ch and ACh. It should also be mentioned that this quantity is really an effective rate constant because the system is complicated by the fact that betaine aldehyde, an intermediate product of the enzymatic oxidation of Ch (equation 4), is also a substrate for choline oxidase. Nevertheless, the values found here for k'_{ME} are quite similar to those found for other enzyme electrode/substrate systems.[2-4]

Following closely the work of Albery, we continue the kinetic analysis by calculating the quantity ρ, shown in (6) below, at higher substrate concentrations, where the current is greater than that described by (5).

$$\rho = \{i/[S]\}/\{i/[S]\}_{[S]\to 0} \tag{6}$$

In the usual procedure,[1] these terms are plotted against the quantity y defined below:

$$y = \{(1/\rho) - 1\}/[S] \tag{7}$$

From this so-called rho plot, it is possible to determine the observed Michaelis-Menten constant K_{ME}[15] for the electrode, since y is also

TABLE I

Kinetic Results for Acetylcholinesterase/Choline Oxidase TTF-TCNQ Microelectrodes

	choline[a]	acetylcholine[b]
k'_{ME} (cm/s)	5.7×10^{-4}	5.8×10^{-4}
K_{ME} (M)	7.9×10^{-5}	1.0×10^{-4}
K_M(M)	3.6×10^{-4}	4.1×10^{-4}

[a]Mean results for 12 electrodes.
[b]Mean results for 16 electrodes.

related to ρ through the following expression[1]:

$$y = (1/K_{ME})\,(1 - \rho k'_{ME}/k'_s) \tag{8}$$

Here k'_s is the rate constant for mass transport of the substrate; this term is often rate-limiting for enzyme electrodes using membranes,[2-4] but it should be very large for the present system. This point is borne out in Figure 5, which shows the rho plots for the data in Figure 3. The results for both Ch and ACh show a straight line with a slope of nearly zero, since for large k'_s, enzyme kinetics are rate-limiting and equation 8 gives a constant value for y ($=1/K_{ME}$); thus the observed Michaelis-Menten constant can be read directly from these plots. The mean results for a large number of electrodes are shown in Table I. When the concentration of the substrate is less than K_{ME}, the measured current is proportional to $[S]$; saturation occurs at concentrations greater than this quantity. Our results indicate that the observed Michaelis-Menten constant for the TTF-TCNQ electrode is approximately an order of magnitude smaller than that found in solution (K_M = 1.2 mM for choline oxidase in solution, with choline as the substrate).[16] It is also interesting to note that although the additional step of converting ACh to Ch does not affect the apparent electrochemical rate constant k'_{ME}, it can slightly alter the observed Michaelis-Menten constant.

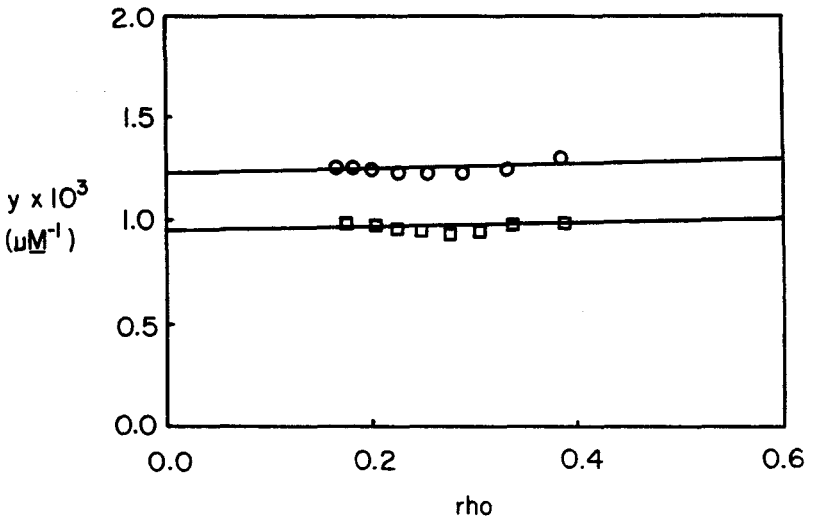

FIGURE 5 Rho plots (equations 6 and 7) [1] for the data shown in Figure 3: (□) choline; (○) acetylcholine.

Finally, it is possible to extend the analysis in order to determine the Michaelis-Menten constant K_M for the homogeneous reaction.[15] The following discussion is valid for systems where there is no membrane over the electrode.[2] In such a case, when the enzyme is unsaturated the current should show a half-order dependence on the substrate concentration:

$$i(\text{unsaturated}) = nAF(Dk_{\text{cat}}[S]/K_M)^{1/2}[E]_{\text{total}} \tag{9}$$

Here D is the diffusion coefficient for the enzyme within the diffusion layer and k_{cat} is the combined rate constant for the reaction of the enzyme-substrate complex to form the enzyme-product complex and its subsequent breakup into product and reduced enzyme[4]; $[E]_{\text{total}}$ is the total concentration of enzyme in the electrode. If the enzyme is saturated, the current is independent of substrate concentration[2]:

$$i(\text{saturated}) = nAF(Dk_{\text{cat}})^{1/2}[E]_{\text{total}} \tag{10}$$

From (9) and (10), one may write the following expression for the homogeneous Michaelis-Menten constant:

$$K_M = \{[S]^{1/2}/i(\text{unsaturated})\}^2 \{i(\text{saturated})\}^2 \tag{11}$$

The first term in this equation can be found from plots such as those shown in Figure 6, where the current has been plotted against the square root of the substrate concentration. Its value is given by the inverse of the slope of the linear region of the curve (for convenience, only the linear, or unsaturated, region is shown in Figure 6). The current in the saturated region can be taken directly from calibration curves like those in Figure 3; we have taken i(saturated) as the maximum measured current in the i vs. $[S]$ plots. The values for K_M resulting from this analysis are shown in Table I, and are in reasonable agreement with the literature value for the choline oxidase/choline system.[16]

The results presented here demonstrate the feasibility of directly detecting the neurotransmitter ACh with a double enzyme-modified TTF-TCNQ microelectrode. Work is presently underway to extend the concentration range of the electrodes (unsaturated region) by coating with a polymer membrane such as Nafion[17] in order to make mass transport of the substrate the rate-limiting step rather than the enzyme kinetics. Preliminary results[18] indicate that such a procedure

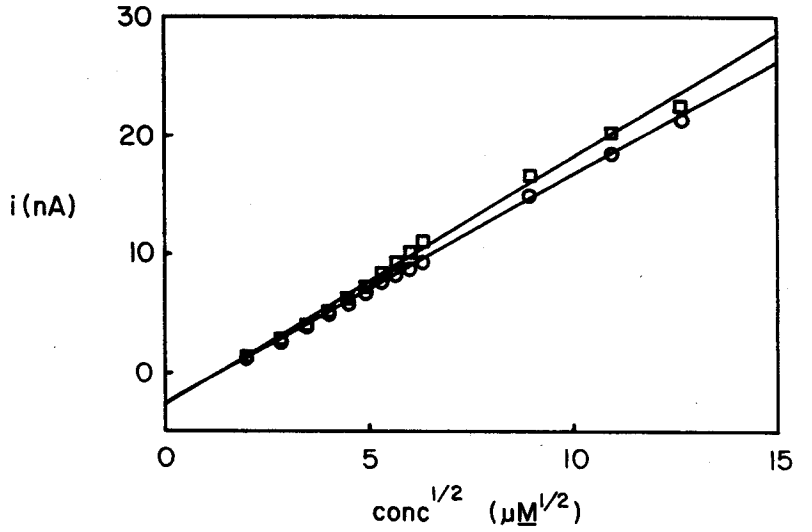

FIGURE 6 Plots of current against square root of substrate concentration for the data shown in Figure 3: (□) choline; (○) acetylcholine.

can increase the range of the electrodes' linear response by approximately an order of magnitude.

Acknowledgment

This research was supported by the National Institutes of Health (NS15841).

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