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Potentiometric Determination of Arylsulfatase Activity Using a Novel Nitrocatechol Sulfate PVC Membrane Sensor

Saad S. M. Hassan* and S. A. M. Marzouk

Department of Chemistry, Faculty of Science, Ain Shams University, Cairo, Egypt

M. M. Abdel-Fattah and G. M. S. M. Shoukry

Department of Biochemistry, Faculty of Science, Ain Shams University, Cairo, Egypt

A novel potentiometric assay method for arylsulfatase enzyme is described based on measuring the initial reaction rate of hydrolysis of 4-nitrocatechol sulfate (4-NCS) substrate under optimized conditions. A monitoring sensor incorporating a PVC membrane with a nickel(II) bathophenanthroline–4-NCS ion-pair complex as electroactive material and 2-nitrophenyl phenyl ether as solvent mediator is developed and characterized. The sensor exhibits fast and stable linear response for 8×10^{-3}–7.5×10^{-6} M 4-NCS with an anionic slope of 58.5 ± 0.2 mV/decade over the pH range 3–6. The sensor is used to follow up the decrease in a fixed concentration of 4-NCS (2×10^{-4} M) as a function of arylsulfatase activity at pH 5.6 and 37 °C. A linear relationship between the initial rate of substrate hydrolysis and enzyme activity holds for 0.2–2.4 IU/mL (SD 2%). Activity measurement of arylsulfatase enzyme isolated from camel liver gives results that compare favorably well with data obtained using the standard spectrophotometric assay method. Significant advantages over many of the previously described spectroscopic methods are offered by the proposed potentiometric technique.

The lysosomal arylsulfatase enzymes (arylsulfatase sulfohydrolase, EC 3.1.6.1) are potentially useful biochemical markers for several types of cancer. An increase of the enzyme activity has been detected in chronic myeloid leukemia, lymphatic leukemia, lymphogranulomatosis (Hodgkin's disease), and cancer of the breast, bladder, womb, and prostate as well as cancer of the skin at various sites.[1–5] Methods in common use for determining arylsulfatase activity generally involve incubation of the enzyme with synthetic sulfated phenolic substrates followed by spectrophotometric and spectrofluorometric rate measurements of the phenoxide ion produced.[6–8]

4-Nitrocatechol sulfate (4-NCS) has proved to be one of the most successful substrates used for spectrophotometric measurement of arylsulfatase activity.[9–12] Inherent disadvantages are the required long incubation reaction time (90–180 min),[9,10] the limitation to unbuffered test solutions,[11] and the inapplicability for continuous monitoring of the enzyme.[12] Nitroquinol sulfate substrate has proved suitable for continuous assay;[13] however, it is unstable in alkaline conditions and its sensitivity is less by a factor of 0.4 than that of 4-NCS.[14] A similar method using 4-nitrophenyl sulfate substrates has been proposed and evaluated by repeated scanning either between two wavelengths or at a constant wavelength in the ultraviolet–visible region.[15] Despite the applicability of the method over a wide pH range, it is not sensitive enough for measuring enzyme activities of less than 30 units.[16]

A spectrofluorometric assay method of measuring arylsulfatase activity has been described based on the use of 4-methylumbelliferyl sulfate substrate, measuring the fluorescence of the hydrolysis product.[17] Because of the insolubility of the substrate and its lack of reactivity, only a 10-fold increase in sensitivity compared with the spectrophotometric methods was gained.[15,17] A more sensitive fluorometric method for measuring as little as 0.6 unit of the enzyme has been suggested that uses 2-naphthyl sulfate substrate.[16] None of the reported kinetic spectrophotometric and fluorometric methods, however, is without limitations. They are generally inapplicable to turbid, colored, and buffered test solutions and samples containing quenching foreign substances. All these drawbacks can be eliminated by using potentiometric techniques.

On the other hand, potentiometric membrane sensors have gained significant utility in the study of various enzyme-catalyzed

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reactions and in the preparation of different biosensors.[18-22] This approach has never been tried for assessment of arylsulfatase activity. In this paper, a kinetic–potentiometric method is developed for simple, rapid, and sensitive measurement of arylsulfatase activity based on the monitoring of its reaction with 4-NCS substrate using a novel 4-NCS poly(vinyl chloride) (PVC) membrane sensor. Initial slope method is used to construct the calibration graph and to measure the enzyme activity over the range 0.2–2.4 IU/mL with a precision of 2%.

EXPERIMENTAL SECTION

Apparatus. All potentiometric measurements were made at 37 ± 0.2 °C with an Orion 720 pH/mV meter using a 4-NCS PVC membrane sensor in conjunction with a single junction Ag/AgCl reference electrode (Orion 90-01) filled with 10% (w/v) potassium chloride. Ross pH electrode (Orion 81-02) was used for all pH measurements. Spectrophotometric measurements were carried out with a Pye Unicam SP-1800 spectrometer at 510 nm using matched 10-mm silica cuvettes. A temperature-controlled (±0.1 °C) water thermostat and a 50-mL double jacketed glass cell were used.

Reagents and Materials. All chemicals were of analytical reagent grade unless otherwise stated, and all solutions were prepared with doubly distilled deionized water. 4,7-Diphenyl-1,-10-phenanthroline (purity ≥ 97%), tetrahydrofuran (THF), and PVC powder were obtained from Aldrich Chemical Co. (Milwaukee, WI). 2-Nitrophenyl phenyl ether (NPPE, purity ~ 99%) was obtained from Eastman Kodak (Rochester, NY). 4-NCS (dipotassium salt) and 4-nitrocatechol (4-NC) were purchased from Sigma Chemical Co. (St. Louis, MO). Arylsulfatase enzymes were isolated from camel liver, fractionated, purified, and characterized. Detailed procedures will be published elsewhere. Arylsulfatase A fraction was used in this study.

4-NCS PVC Membrane Sensor. A 0.1-g portion of 4,7-diphenyl-1,10-phenanthroline (bathophenanthroline, bphen) was dissolved in 20 mL of 95% (v/v) ethanol/water mixture. The solution was mixed with 1.0 mL of 0.1 M nickel(II) chloride solution and stirred for 5 min, and then 5 mL of 0.1 M aqueous 4-NCS (dipotassium salt) solution was added. A yellowish-green precipitate was formed, filtered off on Whatman filter paper No. 42, washed with cold deionized water, dried at room temperature for 24 h, and ground to a fine powder. Elemental analysis and infrared data confirmed the formation of the complex [Ni(bathophenanthroline)$_3$][$C_6H_4NSO_7$]$_2$. A PVC membrane was prepared and used for construction of the sensor as previously described.[23-25] An internal reference solution consisting of a 1:1 mixture of 1×10^{-2} M aqueous dipotassium 4-NCS and KCl was used. The sensor was conditioned by soaking in 1×10^{-1} M aqueous dipotassium 4-NCS solution for 10 min and stored in the same solution when not in use.

Potentiometric Determination of Arylsulfatase. A mixture of 0.10 mL of 1×10^{-2} M 4-NCS and 4.80 mL of 0.1 M sodium acetate–acetic acid buffer of pH 5.6 was transferred to a 50-mL double jacketed reaction cell thermostated at 37 ± 0.1 °C. The 4-NCS PVC membrane electrode, in conjunction with a single junction Ag/AgCl reference electrode, was immersed in the solution. After a constant potential reading was obtained, 100-μL aliquots containing 0.2–3.6 IU of arylsulfatase enzyme were added. The rate curves were plotted, and the maximum initial rates of potential change expressed in millivolts per minute were graphically determined by using the rate portion of the curve. The initial rates were plotted as a function of the enzyme activities. The calibration graph was used for subsequent measurements of unknown enzyme activity. A blank experiment was carried out under similar conditions without the enzyme.

RESULTS AND DISCUSSION

Arylsulfatase catalyzes the hydrolysis of 4-NCS according to eq 1:

$$\underset{\text{(4-NCS)}}{\text{4-nitrocatechol sulfate}} + H_2O \xrightarrow{\text{EC 3.1.6.1}} \underset{\text{(4-NC)}}{\text{4-nitrocatechol}} + H_2SO_4 \quad (1)$$

The enzyme activity can be thus evaluated potentiometrically by determining the increase in concentration of the liberated desulfated residue (4-NC) or the liberated inorganic sulfate ions or alternatively by monitoring the decrease in the concentration of the unhydrolyzed substrate (4-NCS). Since no reliable sensor for sulfate ion is available to date to be used for measuring the liberated inorganic sulfate ion, attempts were made to develop a suitable membrane sensor to determine the concentration of the substrate or its hydrolyzed product as a function of enzyme activity.

Nature and Performance Characteristics of the Monitoring Sensor. A preliminary investigation indicated that nickel tris(bathophenanthroline) cation reacts with 4-NCS anion (the substrate) to form a water-insoluble 1:2 ion association complex of the type [Ni(bphen)$_3$][4-NCS]$_2$. Attempts to prepare an ion association complex containing 4-NC (the reaction product) as a counteranion, however, were not successful. The reaction of nickel tris(bathophenanthroline) with 4-NC gave a water-soluble colored reaction product with a maximum absorption at 496 nm. The color of the reaction product exactly matched that of the product of direct reaction of 4-NC with Ni(II), and the product was precipitated by addition of quaternary ammonium salts. This indicates a high affinity of 4-NC to complex nickel and to displace bathophenanthroline to form [Ni(4-NC)$_2$]$^{2-}$ species.

A plastic membrane incorporating [Ni(bphen)$_3$][4-NCS]$_2$ as an electroactive material and NPPE plasticizer was prepared using a casting solution of the composition 28:2:70% (w/w/w) of PVC/ion-pair complex/NPPE. The electrochemical performance characteristics of the sensor were systematically evaluated according to IUPAC recommendations,[26] and the results are given in Table 1. The calibration curve obtained with the 4-NCS PVC membrane sensor at pH 3–6 shows a near-Nernstian and reproducible response in the range of 7.5×10^{-6}–8×10^{-3} M 4-NCS with an anionic slope of 58.5 ± 0.2 mV/M·decade (Figure 1).

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Table 1. Response Characteristics of 4-NCS PVC Membrane Sensor

parameter	value
slope (mV/decade)	58.5 ± 0.2
intercept (mV)	−185.5
correlation coefficient (r)	0.997
lower limit of linear range (M)	7.5×10^{-6}
lower limit of detection (M)	4.5×10^{-6}
response time for 1×10^{-3} M (s)	5
recovery time for 1×10^{-3} M (s)	20
working acidity range (pH)	3–6

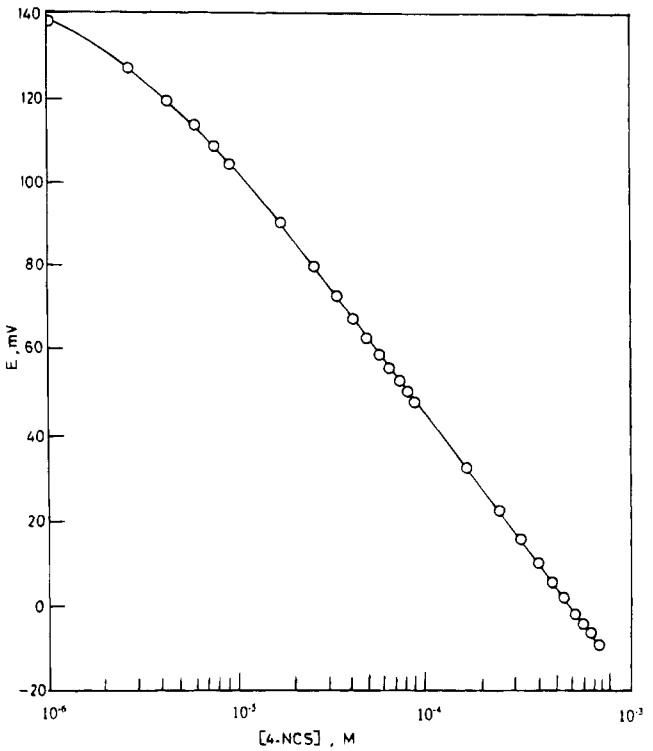


Figure 1. Response graph for the 4-NCS PVC membrane sensor.

Potential Response and Stability of the Sensor. The dynamic response time of the sensor was tested for 1×10^{-5}–1×10^{-3} M 4-NCS solution. The time required for the sensor to reach values within ±0.5 mV from the final equilibrium potential after increasing the 4-NCS concentration level 10-fold is fairly short. It reaches 95% of its final steady potential after 5 s for [4-NCS] ≥ 1×10^{-4} M and 15 s for [4-NCS] < 1×10^{-4} M.

The potential stability displayed by the 4-NCS sensor for consecutive measurements of 1×10^{-5}–1×10^{-3} M standard 4-NCS solution in the same day did not vary by more than ±1 mV (n = 10). Changes in the calibration slopes did not exceed ±0.3 mV/decade change of concentration. The long-term reproducibility and stability of the potential was also evaluated by determining replicate calibration graphs (n = 10) over a period of 3 weeks. During this period, the sensor was stored and conditioned in 1×10^{-2} M 4-NCS solution and thoroughly washed with water between measurements. The slope of the calibration graphs remained practically constant within ±1–2 mV/M·decade over this period. The detection limit, linear range, and response time of the sensor were almost constant.

Effect of pH on the Sensor Response. The effect of the pH of the 4-NCS test solutions (1×10^{-3} and 1×10^{-4} M) on the sensor response was investigated by following the variation of potential with the pH change over the range 3–10. The results obtained are illustrated in Figure 2. These plots are characteristics of the pH-dependent equilibrium between the mono- and divalent anionic species of 4-NCS over different pH values. The sensor's potential is almost constant over the pH range 3–6, displaying a slope of 58.5 ± 0.2 mV/M·decade, attributed to the predominant presence of monovalent 4-NCS anion. At pH > 6, the potential rapidly increases due to the formation of the divalent 4-NCS anion. A slope of 30 ± 0.3 mV/M·decade is consistently obtained at pH ≥ 9.5 due to the complete conversion of the substrate monovalent anionic species into the divalent anionic form (eq 2).

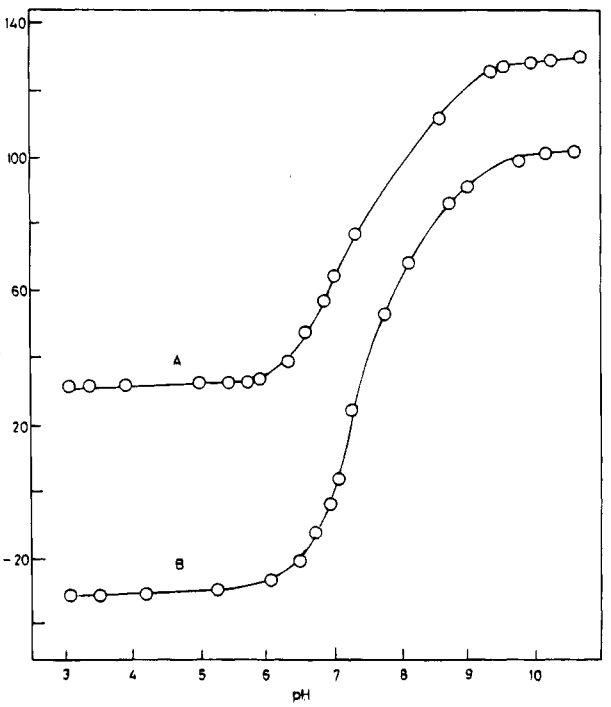
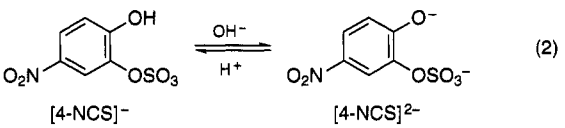


Figure 2. Effect of pH on the response of 4-NCS PVC membrane sensor at different substrate concentrations: (A) 1×10^{-4} and (B) 1×10^{-3} M 4-NCS.



(2)

Since the present enzymatic reaction proceeded at an optimum pH of 5.6, all subsequent measurements were made in slightly acidic media. Acetate buffer of pH 5.6 provided the most appropriate conditions for maximum enzyme activity, fast sensor response, and high calibration slope.

Enzymatic Reaction Conditions. To evaluate the feasibility of using the 4-NCS sensor for monitoring the rate of arylsulfatase-catalyzed reaction, the potentiometric selectivity ratio of 4-NCS over 4-NC was measured by the separate solutions method,[18,24] using 1×10^{-3} M solutions at pH 5.6. The selectivity coefficient for 4-NCS over 4-NC was found to be approximately 500. Since the initial rate method was used for monitoring the consumption of 4-NCS substrate, interference from the reaction product 4-NC was significantly minimal. A similar approach has been described by Llenado and Rechnitz[27] for determining rhodanase activity by

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following the consumption of the cyanide ion substrate with a solid state cyanide ion-selective electrode. In the present study, reaction conditions such as temperature, pH, type of buffer, and substrate concentration were optimized for the assay of arylsufatase activity.

Effect of Substrate Concentration. The Michaelis–Menten equation gives the relation between the initial rate of the enzymatic reaction and the substrate concentration,[28]

$$-\frac{d[S]}{dt} = \frac{K[Z][S]}{K_m + [S]} \quad (3)$$

where [Z] and [S] represent the concentrations of the enzyme and substrate, respectively, K is the rate constant for disproportionation of the enzyme–substrate complex into free enzyme and product, K_m is the Michaelis constant, and t is the time.

The potential E of the 4-NCS sensor used for monitoring the reaction is given by the Nernst equation:

$$E = E_0 - \text{slope} \log [S] \quad (4)$$

Differentiating eq 4 with time gives

$$\frac{dE}{dt} = -\text{slope} \frac{1}{[S]} \frac{d[S]}{dt} \quad (5)$$

Substituting for $d[S]/dt$ from eq 3 gives

$$\frac{dE}{dt} = \text{slope} \frac{K[Z]}{(K_m + [S])} \quad (6)$$

Equation 6 shows that the initial potential change is inversely proportional to the substrate concentration at a constant enzyme activity. Aliquots of 10–500 μL of 1×10^{-2} M 4-NCS solutions were diluted to a final volume of 4.95 mL with 0.1 M acetate buffer (pH 5.6) to give solutions with substrate concentrations in the range 2×10^{-5}–1×10^{-3} M. A 50-μL aliquot of arylsulfatase enzyme (≡0.033 IU) was added, and the potential change was measuerd. It may be mentioned that enzyme activities are commonly determined by methods involving saturation of the enzyme with the substrate. In this study, however, where the K_m value of the enzyme is 2×10^{-3} M, the use of a saturating substrate concentration was inconvenient for measuring accurately small changes of the substrate in the presence of high initial substrate background.

On the other hand, the use of 4-NCS concentrations in the range of 1×10^{-3}–1×10^{-4} M secured sensitive measurement of the initial reaction rate in accordance with eq 6. Lower substrate concentrations (2×10^{-5}–1×10^{-4} M) did not significantly increase the measured initial reaction rate ($\Delta E/dt$), probably due to the decrease of both the real reaction rate ($-d[S]/dt$) and the sensitivity of the sensor at low substrate concentrations. A 2×10^{-4} M 4-NCS solution was used in all subsequent measurements. This compromise concentration level offered a measurable change in the reaction rate at low enzyme activity, a better linearity of the calibration plot, and a fast sensor response.

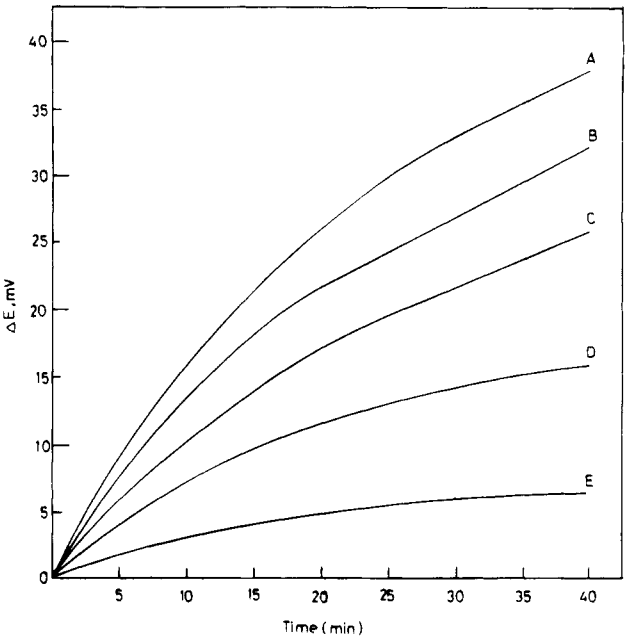

Figure 3. Effect of 4-NCS concentration: (A) 1×10^{-4}, (B) 2×10^{-4}, (C) 3×10^{-4}, (D) 5×10^{-4}, and (E) 1×10^{-3} M on the rate curves using 0.033 IU arylsulfatase enzyme at pH 5.6 and 37 °C.

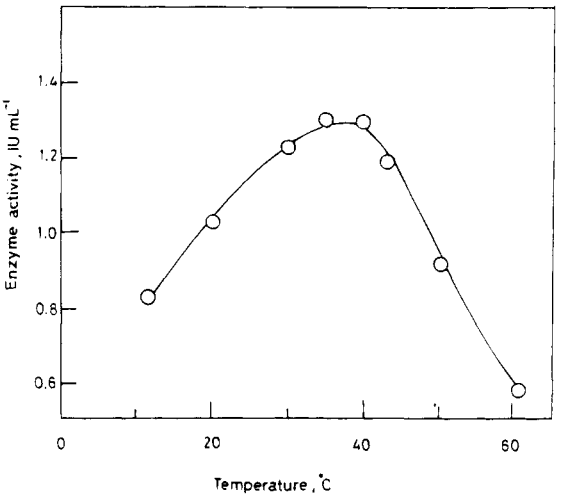

Figure 4. Effect of temperature on the activity of arylsulfatase using 2×10^{-4} M 4-NCS substrate at pH 5.6.

Figure 3 shows the E–t recordings of the reaction. The shapes of these curves are almost identical with those previously obtained[27] for determination of rhodanase activity by following the consumption of cyanide ion substrate (sensor slope < 0), and also with the pattern of E–t recordings known for kinetic reactions involving a rapid reversible formation of an intermediate species.[29]

Effects of Temperature and pH on the Rate of Reaction. The temperature dependence of the enzymatic reaction was also investigated in the range 10–60 °C. The results obtained are shown in Figure 4. The reaction rate reached its maximum at 30–42 °C. The pH profile at 37 °C under similar conditions showed a maximum reaction rate over the pH range 4.9–5.6 (Figure 5). Since the proposed sensor displayed maximum and fast response for 4-NCS monovalent anion at pH 3–6, all subsequent measurements of the enzymatic reaction rate were carried out in 0.1 M acetate buffer of pH 5.6 at 37 °C.

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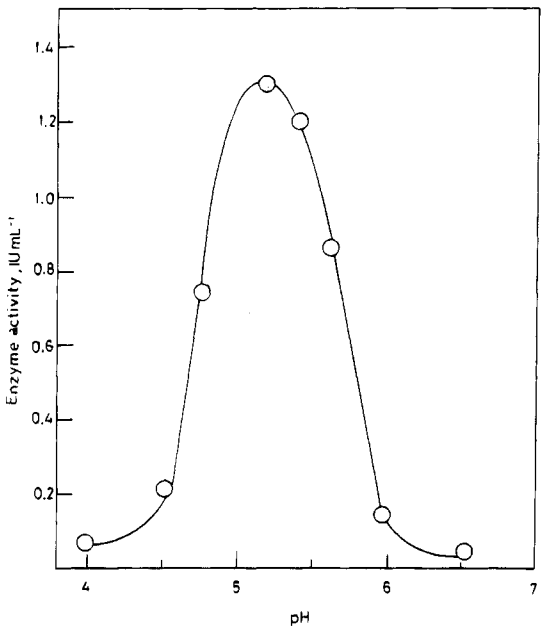

Figure 5. Effect of pH on the activity of arylsulfatase using 2 × 10^{-4} M 4-NCS substrate at 37 °C.

Table 2. Determination of Arylsulfatase Activity (IU/mL)[a] Using 4-NCS PVC Membrane Sensor (Potentiometry)

	found	
added	spectrophotometry[b]	potentiometry[c]
0.2	0.198 ± 0.021	0.196 ± 0.006
0.4	0.397 ± 0.018	0.406 ± 0.008
0.8	0.807 ± 0.023	0.795 ± 0.012
1.2	1.200 ± 0.032	1.210 ± 0.022
1.8	1.799 ± 0.027	1.821 ± 0.030
2.4	2.391 ± 0.055	2.372 ± 0.045

[a] Average of five measurements. [b] Measured by the standard colorimetric method. [c] Measured using 4-NCS PVC membrane sensor.

Enzyme Assay. Arylsulfatase enzyme isolated from camel liver (activity ~ 500 IU/mg of protein) was diluted with 0.1 M acetate buffer to give a series of various activities. The activity of each solution was determined by the present potentiometric technique and the standard spectrophotometric procedure. The results are given in Table 2. The response of 4-NCS PVC membrane toward different enzyme activities was followed under the optimal reaction conditions. A linear dependence of the initial reaction rate ($\Delta E/\Delta T$) on the enzyme activity was observed over the activity range of 0.2–2.4 IU/mL (Figure 6). At higher enzyme activity, however, a slight curvature was noticed. The accuracy and precision of the method were calculated from 10 repetitive analyses made on 1 IU/mL of arylsulfatase; the average precision was 2%.

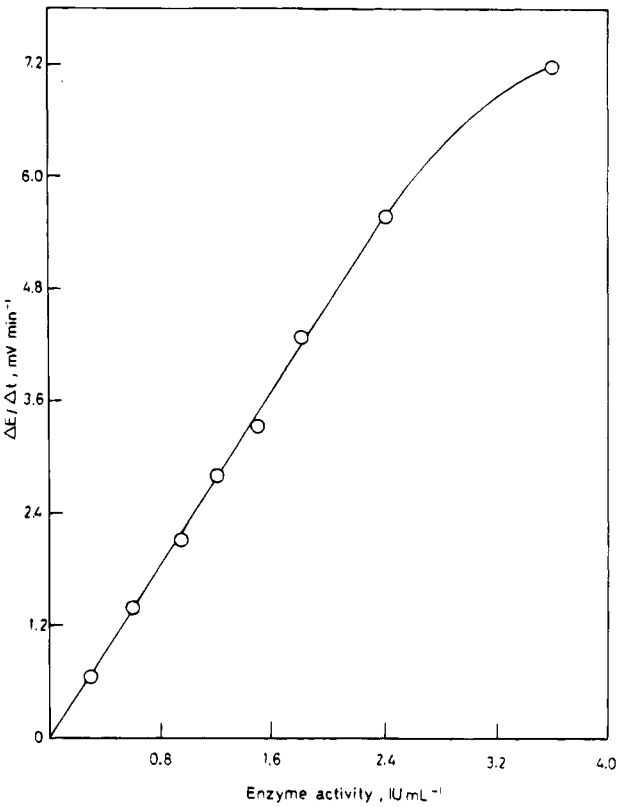

Figure 6. Initial rate of hydrolysis of 2 × 10^{-4} M 4-NCS substrate as a function of arylsulfatase activity at pH 5.6 and 37 °C.

CONCLUSION

The present potentiometric assay method for arylsulfatase activity is simpler and more convenient than many of the earlier spectrochemical techniques. The latter methods require a long incubation reaction time (~1 h)[10] followed by termination, phenoxide formation, and color or fluorescence measurements. The present potentiometric method, however, is fast (5 min), readily applicable to colored and turbid solutions, usable for continuous monitoring, and adaptable for routine and automated analyses. The detection limit offered by the present potentiometric method (0.05 IU/mL) is lower than those obtained by some instrumental methods in current use.[15,16]

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