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Ultramicro enzyme assays in a capillary electrophoretic system

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ABSTRACT

This paper describes an ultramicro method for achieving enzyme assays. Enzyme saturating concentrations of substrate, coenzyme when appropriate, and running buffer were mixed and used to fill a deactivated fused-silica capillary in a capillary zone electrophoresis apparatus. The enzyme glucose-6-phosphate dehydrogenase was injected by either electrophoresis or siphoning and mixed with the reagents in the capillary by electrophoretic mixing. Enzyme activity was assayed by electrophoresing the product, reduced nicotinamide adenine dinucleotide phosphate, to the detector where it was detected at 340 nm. Under constant potential, the transport velocity of enzyme and the product was generally different. This caused product to be separated from the enzyme after it was formed. Because product formation was much faster than the rate of enzyme-product separation, product accumulated. The amount of accumulated product was inversely related to operating potential. In the extreme case, the operating potential was zero. Zero potential assays were generally carried out by electrophoresing the enzyme partially through the capillary and then switching to zero potential. This capillary was left at zero potential for several minutes to allow additional product to accumulate. After this additional amplification step, potential was again applied and the product transported to the detector. Product formed under constant potential appears as a broad peak with a flat plateau. When the voltage is switched to zero at intermediate migration distance, a peak will be observed on top of this plateau. Either the eight of the plateau or the area of the peak may be used to determine enzyme concentration. The lower limit of detection was $4.6 \cdot 10^{-17}$ mol of glucose-6-phosphate dehydrogenase.

INTRODUCTION

Enzymes are often identified and quantitated by measuring their biological activity, *i.e.* their catalytic behavior. The Michaelis–Menten equation

$$v = (V_{max}[S])/(K_m + [S]) \quad (1)$$

shows that the initial reaction rate of a single substrate with an enzyme is related to three variables; the maximum velocity (V_{max}) of the enzyme at substrate saturation, substrate concentration [S], and a rate constant (K_m) unique to each enzyme. At high concentrations of substrate, v will approach V_{max} and remain constant until either (i) substrate depletion begins to occur or (ii) sufficient product accumulates to cause product inhibition. Short reaction times circumvent both of these problems. This equation shows that an enzyme may be assayed in two ways by measuring either the rate of product formation or the amount of product formed in a fixed time. To assay an enzyme by either of these techniques requires three steps: initiation of the reaction by rapid mixing of the reactants, a period of incubation during which product accumulates, and a method for measuring the amount of product formed.

There are several problems associated with assaying enzymes in very small volumes of liquid, particularly in the case of enzymes that have been separated in a capillary electrophoretic system. The first is to mix the reactants rapidly without perturbing the separation. One technique is to attach a post-column reaction detector to the capillary [1] as has been done in liquid chromatography [2–4]. Substrate is added to the system through a mixing-tee at the end of the electrophoresis column and the reaction mixture is pumped into a second capillary.

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Product detection is achieved at the end of the second capillary with a conventional capillary electrophoresis detector. Because the transit time between the mixing-tee and detector is constant, this system approximates a fixed time assay.

A second problem is to minimize band spreading during the mixing and incubation phases of the reaction. The challenge in post-column reaction detectors attached to a capillary separation system is to blend nanoliter streams of eluent and substrate within a few seconds and transport the reactants through a laminar flow system to the detector with minimal band spreading.

Still another problem is how to deal with enzymes that are dilute or have a low turn-over number. Longer incubation times are required to accumulate sufficient product for detection. It is very difficult to achieve incubation times of more than a few minutes in post-column reaction detectors [5]. To do so requires either very long reactors or stopped-flow.

This paper reports a new technique for achieving enzyme assays in a capillary electrophoretic systems using glucose-6-phosphate dehydrogenase (G-6-PDH, EC 1.1.1.49) as a model enzyme. G-6-PDH has be found in almost all animal tissues and microorganisms in which it catalyzes the first reaction in the hexose monophosphate shunt pathway. Since the erythrocyte lacks the citric acid cycle, it depends on the pentose phosphate pathway for its only supply of NADPH, which is required to maintain the intracellular concentration of reduced glutathione. Deficiency of G-6-PDH is an inherited trait, for which more than 80 variants of the enzyme have been described. The clinic biochemistry of G-6-PDH deficiency is of great importance and has been extensively reviewed [6].

THEORY

Enzymatic catalysis may be portrayed as the reaction of an enzyme (E) with a substrate (S) to form an enzyme–substrate complex (ES) which then decomposes to a product (P) and regenerates the enzyme.

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E + P \qquad (2)$$

Depending on the charge characteristics of these substances, they may have different electrophoretic mobilities. This simple fact could be of great use when carrying out ultramicro enzyme assays. Assuming that an enzyme has a net charge of +10, the substrate a net charge of −2, and the product a net charge of −1, the electrophoretic mobility of the enzyme would be much greater than that of the substrate or product and in the opposite direction. A band of enzyme could be made to rapidly overtake a band of substrate by applying potential across the system. The use of electrophoretic transport to merge reagents is a form of mixing. This electrophoretic mixing has several advantages. First, there is little or no dilution when two zones are electrophoretically mixed. Simple diffusion is the only force working to spread and dilute bands in electrophoretic systems [7]. The second advantage of this approach is that turbulence is not required for mixing and band spreading will be minimal. The only exception would be in systems that are dominated by electro-osmotically driven turbulent flow. A third positive feature of electrophoretic mixing is that mixing may be achieved in seconds when the bands or zones are small.

Following mixing, most of an enzyme will be sequestered in the enzyme-substrate (ES) complex. In the example given above, the charge of ES would be the net of E + S, *i.e.* 10 − 2 = 8. The fact that the electrophoretic mobility of the ES complex can be different than either E or S is well known in affinity electrophoresis [8,9]. Techniques have even been developed to determine binding constants based on the differential electrophoretic mobility of the reactants [10]. Because enzyme assays are carried out under substrate saturating conditions, we will assume in the remainder of this discussion that the electrophoretic mobility of the enzyme is that of ES.

Subsequent to the mixing of reactants, incubation in an electrophoretic system could be carried out in two ways. One would be in the zero potential or stopped-flow mode. At zero potential, neither electrokinetic nor electrophoretic transport occurs and the reactants would stay mixed in a single zone where product would accumulate. This corresponds to a fixed time reaction in conventional enzyme assays. A second method would be to carry the assay out under constant potential. In this model, the reactants would be mixed and separated from prod-

uct continuously. Product accumulation and the possibility of an assay would seem to be precluded by continuously separating the enzyme from product. However, enzyme catalysis occurs orders of magnitude faster than the rate of separation. In essence, enzyme assays carried out under constant potential correspond to short, fixed time assays. Product formation on a molar basis could be 10^2–10^4 times greater than the amount of enzyme, depending on the turn-over-number of the enzyme and the potential applied across the zone.

Product detection in the electrophoretic systems described above could be achieved in several ways. In the first, the incubation chamber and optical part of the detector could be juxtapositioned in a manner that would allow absorbance measurements to be made on the incubation mixture. This would be technically difficult and would probably introduce large measurement errors in microsystems. Detection systems that image the whole capillary would probably be the only way detection could be achieved in this manner [11]. A second method would be to transport the product to a fixed detector by some combination of electrophoretic and electrokinetic transport. This approach would adapt readily to capillary electrophoretic (CE) systems. The negative feature of this product transport solution is that some band spreading would occur during transport. Because CE systems produce more than 10^5 theoretical plates, this will probably be a small problem.

An enzyme assay could be carried out in the following way. A surface-deactivated capillary would be filled with an enzyme saturating concentration of substrate, buffer, and all the ingredients necessary for the enzyme assay. Enzyme would be introduced as in any CE system; either by suction or by electrokinetic injection. Subsequent application of potential to the capillary would mix the reactants and allow the reaction to start. As noted above, product and enzyme-substrate complex would be transported through the capillary at different velocities in all but rare cases. Product formation would continue until the enzyme exits the system. In all of the systems discussed below it is assumed that (i) there will be some small negative charge on the walls of the fused-silica capillary, (ii) this negative charge would produce electro-osmotic flow that transports liquid from the anodic to the cathodic end of the capillary. (iii) capillaries would have been sufficiently deactivated that adsorption of enzymes to the capillary walls does not occur, (iv) the enzyme does not absorb at the wavelength chosen for product detection, and (v) assays will be carried out in a conventional capillary zone electrophoresis system.

The theoretical elution profile of an ES–P system in which the transport velocity of ES > P would be as shown in Fig. 1A. Because the ES complex migrates at a higher velocity than the product, the first product detected at point A in the electropherogram will be that which was formed as the enzyme migrated past the detector. In contrast, product detected at point B is that which was formed when the enzyme was introduced at the inlet of the capillary. The peak or spike at B is an artifact that is the result of product formed between the time that enzyme was introduced into the capillary and potential was applied. During this time the capillary was at zero potential and product accumulated. The electropherogram for an ES–P system in which the transport velocity of P > ES would be the opposite (Fig.

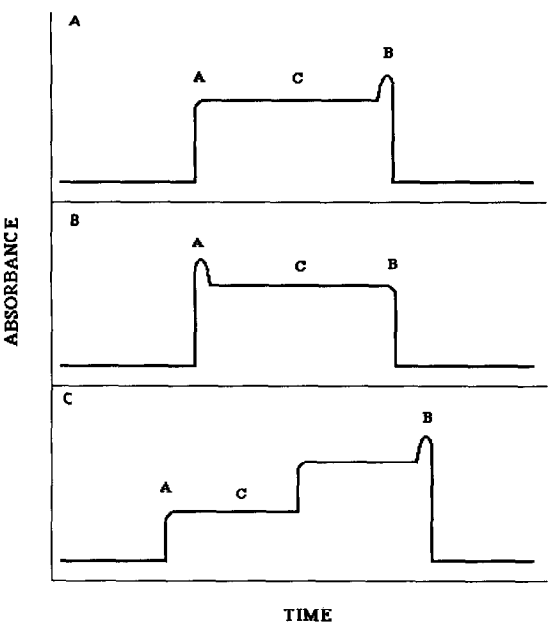

Fig. 1. Predicted models showing various electropherograms in capillary electrophoretic enzyme assay. The moving velocities are (A) ES > P and (B) P > ES. A multiple isoenzyme form is shown in (C) with the moving velocity of their common product smaller than those of these isoenzymes.

1B). The first product to reach the detector at point A would be that which was formed at the capillary inlet. The last product arriving at point B would be that which was formed as the enzyme passes the detector. The transport time of the enzyme and relative transport velocities of ES and P are easily recognized in the electropherograms. When the artifact peak elutes last at point B, the transport velocity of ES > P and point A is the transit time of the enzyme. The opposite is true in the case where the transport velocity of P > ES. In the case of isoenzymes it is expected that one would see multiple peaks similar to the illustration in Fig. 1C.

The height of the plateau above the baseline indicated by C in Fig. 1 will be directly proportional to enzyme concentration at constant potential. Sensitivity will be inversely related to potential, highest sensitivity being obtained at low potential where the greatest amount of product can accumulate before the separation of ES and P. The extreme case would be at zero potential. Switching to zero potential for a fixed time intermediate in the transit of enzyme through the system enables more product to be accumulated and sensitivity to be enhanced. The idea is to stop the power supply before the enzyme passes the detection window and let the enzyme catalyze the reaction to accumulate NADPH in its position. When the power is turned on again the enzyme will be separated from the formed NADPH and a peak will be seen on the plateau and indicate the amount of NADPH formed during the accumulation period. An illustration of the use of this programmed potential during the course of an assay to enhance sensitivity is shown in Fig. 2. Either peak height or area above the constant potential plateau can be used in quantitation.

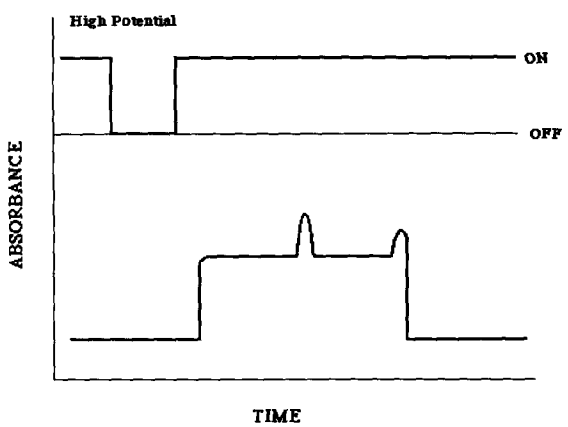

Fig. 2. Proposed potential program and the corresponding electropherogram.

EXPERIMENTAL

Instrumentation

Assays were carried out on two systems. The first was an ISCO 3850 CE system (Instrument Specialties, Lincoln, NE, USA) which was interfaced to a personal computer using Inject software to collect and process data. This software was obtained from Bioanalytical Systems (Lafayette, IN, USA). The second CE system was in-house design [14]. Polyamine-coated, fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) of and 35–60 cm × 50 μm I.D. × 360 μm O.D. were used to prepare the columns. The separation lengths were varied from 15–40 cm. Detection was achieved with a variable-wavelength UV absorbance detector (Model V4, Instrument Specialties). Protein elution was monitored at 200 nm and the product NADPH at 340 nm. The neutral marker mesityl oxide was detected at 254 nm. Strip chart recordings were obtained with a Linear 2000 (Linear, Reno, NV, USA) recorder.

Materials

G-6PDH, G-6-PDH reagent and G-6-PDH substrate solutions were purchased from Sigma (St. Louis, MO, USA). Reagents were prepared and the assay carried out according to the literature [12]. Ethyleneglycol diglycidylether (EGDE), 3-glycidoxypropyltrimethoxysilane (GOX), 1,4-Diazabicyclo[2.2.2]octane (DABCO), mesityl oxide, solvents and buffers were obtained from Aldrich (Milwaukee, WI, USA). Buffers were prepared with deionized, doubly distilled water.

Capillary Coating

An epoxy based coating was applied to the fused-silica capillaries used in this study [13,14]. Fused-silica capillaries were activated with 1.0 *M* NaOH prior to derivatization with GOX. The GOX-bonded phase was further crosslinked with EGDE using DABCO as the catalyst. Non-bound monomer and oligomers were forced out of the column with pres-

surized nitrogen and the column was washed with methanol.

Electrophoresis procedures

Protein samples were injected by siphoning. The inlet end of the capillary was inserted into the protein sample and raised about 5 cm for 5 s. Neutral marker was introduced into the capillary in the same way. The running buffer contained all the reagents necessary to assay G-6-PDH. Assay reagents were reconstituted according to the reagent supplier (Sigma). The buffer solution contained 0.7 mmol/l glucose-6-phosphate (G-6-P), 0.5 mmol/l of the coenzyme nicotinamide dinucleotide diphosphate (NADP) and 4 mmol/l maleimide, in addition to a stabilizer and lysing agent. Operating current was controlled within the range 35–50 μA by limiting the applied potential. In no case was a capillary operated above 60 μA. All assays were carried out at ambient temperature without temperature control. Capillaries were cleaned by flushing with 0.01 *M* sodium hydroxide, doubly distilled water, and then the working buffer solution.

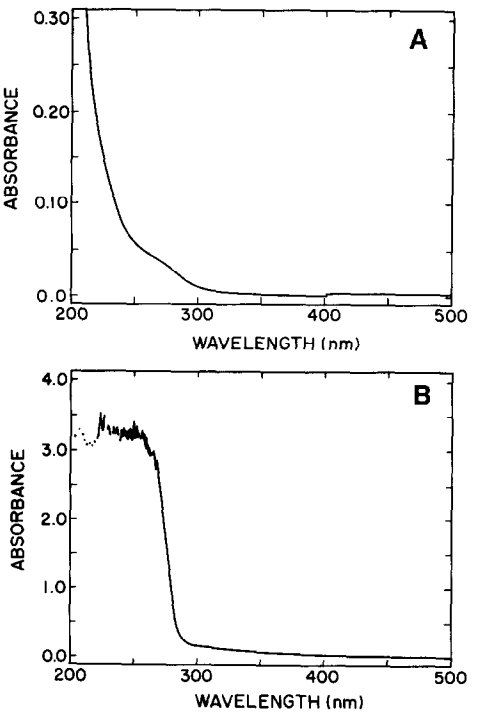

Fig. 3. The absorbance as a function of wavelength, in (A) G-6-PDH, (B) running buffer.

RESULTS AND DISCUSSION

Assay system

G-6-PDH, also referred to as D-glucose-6-phosphate:NADP oxidoreductase, was chosen to examine ultramicro enzymes assays because this enzyme may be readily assayed spectrophotometrically [15]. G-6-PDH oxidizes G-6-P to 6-phosphogluconate (6-PG) while reducing NADP to its reduced form NADPH in the presence of G-6-P.

$$\text{G-6-P} + \text{NADP}^+ \xrightarrow{\text{G-6-PDH}} \text{6-PG} + \text{NADPH} + \text{H}^+ \quad (3)$$

The absorbance spectrum of the product NADPH is uniquely different than that of either the assay reagents or the enzyme (Figs. 3 and 4) and may be used to monitor the reaction [15]. It is seen that NADPH has an adsorption maximum at 340 nm ($\varepsilon = 6.22 \cdot 10^6$ cm^2/mol). The enzyme 6-phosphogluconate dehydrogenase (6-PGDH) can interfere, such as in serum samples contaminated with 6-PGDH from erythrocytes. In the presence of 6-PGDH, 6-phosphogluconic acid may be further oxidized to produce a second mole of NADPH. Addition of maleimide to the incubation mixture will inhibit 6-PGDH.

It is necessary to eliminate protein adsorption in fused-silica capillaries used for enzyme assays. Enzyme immobilized by adsorption at the capillary

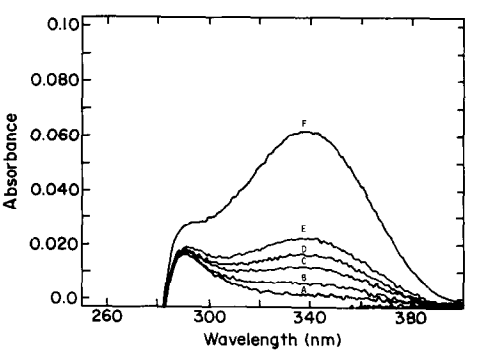

Fig. 4. The absorbance of the reaction system around 340 nm increases as a function of time. (A) 2, (B) 4, (C) 6, (D) 8, (E) 10 and (F) 28 min.

wall causes a series of problems including (i) mixed homogeneous and heterogeneous catalysis, (ii) errors in quantitation from a reduction in product yield, and (iii) increased signal background in subsequent assays. Deactivation was achieved by using a covalently bonded epoxy polymer layer [13,14]. This coating has been shown to give more than 95% recovery of proteins in capillary zone electrophoresis (CZE). Electro-osmotic flow in these deactivated capillaries is substantially reduced and negatively charged species, such as NADPH, can require 20–30 min pass to through 30-cm capillaries. Because G-6-PDH has a relatively high p*I* value, it is transported more quickly. The electrophoretic mobilities of G-6-PDH and NADPH were found to be 5 and 18 min, respectively (data not shown).

Assay protocol

A CZE system was used in which the buffer tanks and capillary had been filled with running buffer containing all the reagents necessary for an assay except the enzyme. Sample enzyme was introduced into the capillary by injection as in CZE and potential applied to mix the reactants. Product formation was measured with a UV detector at 340 nm. Assays were carried out in two ways: (i) a constant potential mode in which the reactants remained under constant potential throughout the course of the assay and (ii) a zero potential mode in which the separation of reactants is stopped during part of the assay.

Constant potential assays. The electropherogram in Fig. 5a of a G-6-PDH assay obtained under constant potential has the general shape predicted in the Theory (Fig. 1) for an enzyme having a greater transport velocity than the product of the reaction

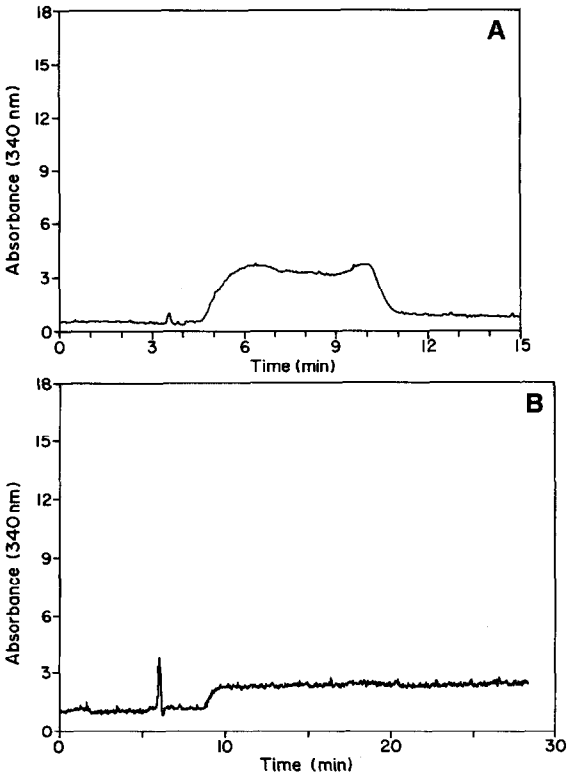

Fig. 5. Typical electropherograms showing the formation of NADPH during the process of G-6-PDH migration through the capillary. (A) Short separation length or high potential situation, (B) long separation length or low potential situation.

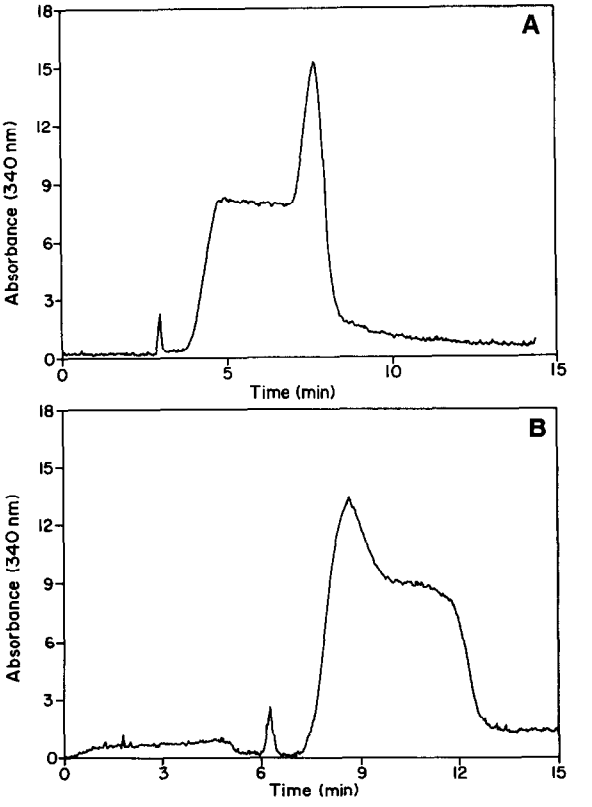

Fig. 6. Electropherograms showing the accumulated peak resulting from the packed reaction at different running times. (A) NADPH accumulated at the beginning before the electrophoresis started, (B) NADPH accumulated just before G-6-PDH passed the detection window.

it catalyzes. This is interpreted to mean that the theory proposed to describe enzyme assays in a capillary electrophoretic system is generally correct. The total time required for the assay in this case is slightly less than 12 min. When a longer capillary was used with a more dilute solution of enzyme it is seen (Fig. 5b) that plateau height is lower and the elution time of the enzyme is longer. Data collection was terminated in this case before the trailing injection artifact peak eluted. The height of the injection artifact peak was variable and of no analytical value. The size of the injection artifact peak was found to be related to the volume and degree of mixing during the injection, enzyme concentration in the sample, and the time elapsed between injection and the start of electrophoresis. An example of allowing several minutes to elapse between injection and the start of electrophoresis is shown in Fig. 6a. For comparison, the sample was injected quickly and the potential dropped to zero for several minutes before the enzyme passed the detector (Fig. 6b). The large peak at approximately 8 min is the result of an interruption of several minutes in the potential, 5 min into the run.

Analysis time could also have been decreased below 12 min without loss in sensitivity by shortening the capillary. The optimum length in terms of minimizing analysis time would be the length required for the product elution curve to plateau. Increasing the potential to shorten analysis time was found to be counter-productive. As predicted, increasing the potential diminishes product accumulation and sensitivity.

The small peak eluting at 3.5 and 6.0 min in Figs. 5a and 5b was not predicted in the Theory. This peak adsorbed at 200 and 340 nm and is thought to be a protein in the sample that either adsorbs at 340 nm or binds NADPH but does not play a role in catalysis.

Zero potential assays. Switching the potential to zero before the enzyme elutes from the capillary allows one to increase the incubation time and concommitantly sensitivity. Zero potential assays were carried out in a 41-cm segment of capillary with the detector set 17.8 cm from the inlet. At 8700 V G-6-PDH reached the detector in approximately 6 min. The potential was interrupted after 3 min and held at zero potential for 5 min after which the potential was returned to 8700 V for product elution. An

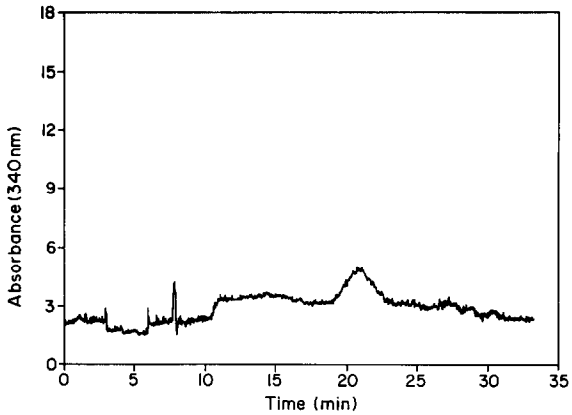

Fig. 7. The accumulation of NADPH by switching to zero potential results in a distinct peak on the plateau. The area of this typical peak may be integrated to give information on enzyme activity.

electropherogram of a sample estimated to contained $4.6 \cdot 10^{-17}$ mol of G-6-PDH is shown in Fig. 7. This estimate is based on the assumption of a 2-nl injection volume. Note the peak at 20 min which resulted from the 5-min zero potential incubation. The dose response curve for G-6-PDH using this assay procedure is shown in Fig. 8. Data for this figure were obtained from Table I. Quantitation is based on determinations of the peak area above the constant potential product plateau in the electropherogram. A solution containing one unit/ml of G-6-PDH is approximately $3 \cdot 10^{-8}$ *M*. No attempt

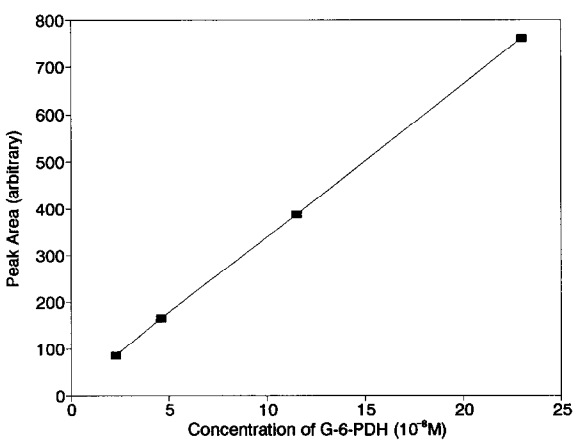

Fig. 8. The relationship between the accumulated NADPH peak area and the concentration of enzyme.

TABLE I

G-6-PDH ACTIVITIES OBTAINED BY CAPILLARY ELECTROPHORESIS ASSAY

Experimental conditions: column, 41 cm GOX-EGDE coated capillary with a separation length of 17.8 cm (50 μm I.D.); power supply, 8700 V, 50 μA; detection, 340 nm; sensitivity, 0.02 a.u.; injection, 10 s.

Assay number	G-6-PDH activity (EU)[a]	G-6-PDH concentration (*M*)	G-6-PDH quantity (mol)	Peak area (arbitrary)
1	0.75	$2.3 \cdot 10^{-8}$	$4.6 \cdot 10^{-17}$	85 241
2	1.50	$4.6 \cdot 10^{-8}$	$9.2 \cdot 10^{-17}$	164 554
3	3.75	$1.15 \cdot 10^{-7}$	$2.3 \cdot 10^{-16}$	386 763
4	7.50	$2.3 \cdot 10^{-7}$	$4.6 \cdot 10^{-16}$	760 207

[a] EU represents enzyme unit as defined in ref. 15.

was made to determine the linear dynamic range of the assay. Based on macroscopic assays it could be two orders of magnitude [15].

CONCLUSIONS

It may be concluded that small quantities of enzyme may be detected in a CZE system by carrying out the assay in the capillary. Assays are based on the fact that transport velocities of the enzyme, reagents, and product(s) are different under applied potential and may be used both to mix the reactants and separate the enzyme from product(s) after catalysis. Product is transported to the CZE detector where product concentration is determined and related to enzyme concentration. The detection limit by this method appears to be three orders of magnitude lower than by conventional methods.

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