The Electrochemical Activity Determination of Trypsin-Like Enzymes

V. A Comparative Study of the Electrochemical Oxidation of the Marker, p-aminodiphenylamine, and of Electrogenic Substrates in Human Plasma, Whole Blood, and Aqueous Solutions

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ABSTRACT

Easily oxidizable aromatic amines can be conveniently used as electrogenic markers for the evaluation of enzymatic activities in whole blood and human plasma dilutions. The analytical procedure is based on the cyclic voltammetric detection of the amine released by thrombin (the tested blood coagulation factor) through an amidolytic cleavage from specific substrates. The relevance of parameters susceptible of interfering with the electrochemical signal have been reviewed using carbon paste and smooth platinum disk electrodes. Results obtained in blood and plasma dilutions are compared with aqueous solutions of the enzyme.

Since the early seventies, the use of chromogenic substrates started a rapid development in the field of the assay of trypsin-like enzymes and of their natural inhibitors. Several enzymes of this type are involved along the overall blood coagulation process. The final step in the blood clotting cascade is the conversion of fibrinogen to fibrin, which leads to the formation of the thrombus. The latter catalytic decomposition is mediated by a protease, thrombin. While thrombin induces the thrombosis, it is normally only present in the precursor form, prothrombin. Beside the clotting enzymes, plasma carries components fated to inhibit their action; thus, the most potent antiprotease associated with thrombin is antithrombin III, which has been definitely shown to play a role in the body's defence against thrombosis.

The quantitative evaluation of antiprotease levels in physiological media is determinant for clinical purposes. From that point of view, the availability of chromogenic substrates provides an access to thrombin and antithrombin activity assay. The principle of the photometric procedure lies in the following: the enzyme is added to a mixture containing the synthetic substrate. Since its chemical structure more or less mimics the C-terminal part of the fibrinopeptide A at the sequence preceding the fibrinogen bond cleaved, thrombin catalyzes the splitting of the chromogenic moiety. The activity of thrombin, or remaining thrombin when antithrombin III levels are assayed, is then proportional to a photometric variable (1).

Most substrates commercially available are designed to release p-nitroaniline (pNA) through amidolytic digestion, which is conveniently monitored photometrically in the visible spectrum region at 405 nm. However, severe limitations restrict the scope of the procedure to colorless, diluted, and homogeneous solutions. Human plasma dilutions fulfill these requirements, but not whole blood; accordingly, antithrombic assays need to be achieved following a centrifugation procedure.

In order to avoid such a more or less traumatic handling of the samples, an electrochemical technique has been recently proposed, so as to permit the assay of trypsin-like enzymes (e.g., trypsin, factors IXa, Xa, kallikrein, thrombin, plasmin, and their inhibitors) in human whole blood. This technique involves the electroanalytical detection of electroactive markers in place of the chromogenic pNA. Several substrates of various specificity have been tested (Fig. 2). D,L benzoylarginyl p-aminodiphenylamide (D,L BAPADA) has been conceived for the assay of trypsin, S-2421, and S-2497 for thrombin and, accordingly, antithrombin III. Each of these substrates bears an easily oxidizable amine, p-aminodiphenylamine (pADA) (2-5).

While the electroanalytical detection of pADA in physiological media can be speculatively thought to be perturbed with respect to aqueous solutions, the aim of this paper is to investigate the influence of parameters which may mediate the electrochemical redox signal in the conditions for an actual assay. Therefore, the results obtained in whole blood and plasma are compared with those of aqueous solutions exempted from physiological fluids. The following variables of potential relevance are examined in the light of the shape and the position of the cyclic voltammogram of the amine: electroactivity of the substrates that have been tested, redox character of the enzymes, pH, and the effect of plasma and blood levels.

Experimental

Instrumentation, cells, and electrodes.—The instrumentation used for the electroanalytical studies was a standard amplifier-based potentiostat/galvanostat PAR Model 179 (Princeton Applied Research) provided with a three electrode configuration. Current-voltage and current-time curves were monitored with a Sefram TGM X-Y recorder and generated with a function generator PAR Model 175. For electroanalytical experiments, a one-compartment cell containing a carbon paste electrode (CPE), platinum wire auxiliary, and aqueous SCE reference was employed. Before each individual run, the carbon paste surface was renewed.

The working electrode circuitry was buffered with a high impedance electrometer PAR Model 178. The smooth platinum disk electrode was embedded in Teflon (area: 0.0314 cm²). Its pretreatment is detailed in the next section. Coulometric n values were obtained with a PAR Model 179 digital coulometer. The vessel consisted of a two-compartment cell in which the
platinum wire auxiliary electrode was in one compartment and the platinum gauze working electrode and aqueous SCE reference were in the other; the two were separated by a porous glass frit. Experiments were conducted in 0.2-7N H₂SO₄ aqueous solutions, in the presence of 5% added methanol or in buffered solutions. These conditions proved to facilitate the dissolution of the reactants. The analytical concentration range for pADA was in all cases 0.1-0.4 mmol.

Solvents and reagents.—Aqueous solutions were prepared from doubly distilled water and reagent grade H₂SO₄ or standard buffer reagents. The p-aminodiphenylamine (pADA) was a commercial product from Fluka (Switzerland), puriss, p.a., which was purified by successive recrystallizations from water/ethanol mixtures. The amine was stored under vacuum in the dark and was no longer used after one week storage. All other materials were reagent grade and were used without further purification.

**Enzymes, plasma, and blood sampling.**—Lyophilized thrombin (51 NIH-U/mg, Lot No. A 3133) was purchased from Hoffman-LaRoche, Basel, Switzerland, and stored in the freezer. Its activity was measured either with chromogenic substrates or with the clotting time method. Before use, aliquots of thrombin were dissolved in tris/NaCl buffer pH 7.45 to a concentration of about 50 NIH-U/ml and preincubated for 30 min in an ice bath. Stock solutions were no longer utilized after 60 min following dissolution. Pipettes and syringes were saturated with the thrombin solution before use. Whole blood was taken from a donor under usual conditions at the Centre National de Transfusion Sanguine (Paris, France): 92 ml blood and 25 ml of a citric acid/monosodium phosphate/glucose solution (3.27% citric acid monohydrate, 2.63% trisodium citrate dihydrate, 1.93% anhydrous monosodium phosphate, and 2.32% anhydrous glucose in 100 ml). The hematocrit was 0.48. Human plasma was prepared by centrifugation at 2000g for 20 min at 4°C and kept frozen at −20°C before use.

**Results and Discussion**

The electrochemical behavior of pADA in aqueous solutions and in dilutions of plasma and whole blood has been investigated with two kinds of electrodes. Mechanistic information has been collected using the carbon paste electrode for the range of potentials attainable with it is fairly extended anodically. It also provides almost zero residual currents. For optimizing the practical purposes, the use of smooth platinum electrodes was studied.

**Aqueous Solutions (Results Obtained with the Carbon Paste Electrode)**

*Influence of pH.*—In the moderate pH range, from 2 to 10, the cyclic voltammogram shows a marked pH dependence. The variation of the peak potential is given by the relation

\[ E_p = 0.06 \, \text{pH} + 0.53V \text{ vs. SCE at 20°C} \]

which holds up to pH 7.5. The slope accounts for the global transfer of an equal number of electrons and protons. The voltammetric analysis of the signal shows the pADA molecule yields the parabenzoquinonediimine form (6). Whereas it is practically constant below pH 7.5, the anodic-to-cathodic peak separation increases and results of increasing anodic irreversibility (Fig. 1). In coincidence, the current ratio \( ip/cp \) decreases. It must be noted that the break occurring in the potential/pH relationship does not arise at the pK for acid-base dissociation of pADA (ca. 9), but two units above, instead. This indicates that the nature of the diffusing species (the protonated pADA) differs from those undergoing the electrochemical oxidation at the electrode surface (the neutral amine). The apparent pK involved would be that of the adsorbed form. Since the irreversibility more markedly affects the cathodic run than the anodic half-cycle, as shown on Fig. 1, it is suggested that the oxidant anodically formed undergoes a chemical conversion becoming preponderant above pH 7.5. The hydrolysis of the diimine may reasonably account for this trend (7).

The increasing irreversibility of the electrochemical signal, however, does not prevent utilizing it as a marker for enzymatic activity determinations; this was checked with amperometric titrations of the amine in solutions covering the pH range mentioned above. As a result, the endpoint procedure can be applied, for example, after blocking the enzymatic kinetic with the addition of acid (5).

*Influence of the sweep rate.*—Simplest voltammetric curves were obtained when scanning rates of 0.2 V/sec were used. At this scanning rate, the peak current \( ip \) was found to be a linear function of p-aminodiphenylamine concentration with an accuracy of ±5% between 10⁻⁶ and 10⁻⁵ M. At lower scanning rates, faradaic and adsorption oxidation peaks of decomposition products of the radicals formed in the first electron uptake (6) were observed at potentials more positive than the oxidation peak of the parent compound.

*Influence of the substrate.*—Adequate substrates for electrochemical determinations should not be electroactive, at least in the potential span where the marker itself is detected. Three peptides were tested: D,L benzoylarginyl p-aminodiphenylamide, S-2497, and S-2421. The benzoylarginyl-p-aminodiphenylamide and S-2497 yielded p-aminodiphenylamine and S-2421 yielded 4-methoxy-2-naphthylamine (Fig. 2). The first peptide differed from S-2497 and S-2421 by bearing a benzoylarginyl group instead of the phenylalanyl-pip-eoclylarginyl group of the latter two.

The results of the electrochemical behavior of these substrates are presented in Fig. 3 and Table I. The study was performed in tris/NaCl buffered solutions at pH 7.45. The cyclic voltammograms of the three substrates are similar; they show two anodic peaks for the 10⁻⁵M level, which gradually merge into one unique symmetrical peak above the 10⁻³M range of concentrations. The associated cathodic peaks are not present in each case. Otherwise, the anodic peak current amplitudes are not linearly related to the concentrations of the depolarizer in the studied range. Rough calculation of the apparent diffusion coefficients leads to values above five times larger than that of the released amine. As concerns a relative insight, the data suggest that the ratio \( ip/cp \) for the three sub-
strates vary in the order of increasing molecular weight and/or adsorption ability, inasmuch as naphthyl groups are more capable of adsorbing than p-diphenylamine groups (8).

These observations indicate that preponderant adsorption controlled processes accompany the irreversible charge transfer reaction, in such a way that the whole peak only reflects a minor contribution of the faradaic component. This is best shown at low concentrations where the second peak ascribed to the faradaic reaction is gradually overlapped in favor of the preceding adsorption peak, in proportion the concentration is increased.

The potentials of the faradaic peaks were compared to those of the respective amines, in solutions of identical composition. The results are reported in Table I. The constant differences which are obtained provide the indication that the loss of the electrons is in each case promoted by the amine linked up with the peptide chain. It may be concluded that the electron withdrawing ability of the latter is essentially equivalent for the three substrates, whichever group substitutes the arginyl radical.

Due to the final levels at which the substrate is required to allow a pseudo first order kinetic to proceed (at least \(10^{-4}\)M), adsorption affects the electrochemical signal of the amine when the potential exploration is pursued past the oxidation peak. This holds especially when consecutive sweeps are effectuated with the same electrode surface. In the case of the carbon paste electrode, however, the surface is renewed before each run. Therefore, although wide potential spans are attainable unless a fixed potential detection is used. The current important with regard to amperometric procedures is envisaged that DMSO combines to dissolve superfluous the paraffin used to elaborate the electrode material. Thus, the increasing irreversibility would account for an etching phenomenon of the electrode surface by diminishing its catalytic properties rather than for an electrokinetic effect occurring in the double layer.

Per se, glycine does not inhibit the reversibility of the voltammetric peak since the \(E_{p2} - E_{p1}\) (1/2) variation remains the same as for acidic solutions; the observed 40 mV correspond to a number of exchanged electrons intermediate between one and two, which is compatible with the mechanism described elsewhere for the actual sweep rate (6). Although glycine shows no significant effect on the reversibility of the electronic transfer, it nevertheless affects the position of the peak potential, rendering it more anodic. Increasing the amount of DMSO acts likewise. In the case of glycine instead, it is probable that the amino acid modifies the structure of the solvent-electrode interface, presumably through local adsorption. Hence, the transfer of the electrons likely occurs under different local pH conditions which would be responsible for the anodic shift. On the other hand, adsorption of glycine molecules might also restrict the effectiveness of the electron surface. It is thus not surprising to note a correlative decrease in the \(i_{pa}/C\) values.

From these partial results, it can be concluded that accurate determinations of peak heights require precise experimental conditions. The potential shift is unimportant with regard to amperometric procedures unless a fixed potential detection is used. The current

<table>
<thead>
<tr>
<th>Substrates</th>
<th>(E_{p2}) (V)</th>
<th>(E_{p1}) (V)</th>
<th>(E_{p2} - E_{p1})</th>
<th>Mean deviation ±5 mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bz-D,L-ARG-0H</td>
<td>1.0</td>
<td>0.6</td>
<td>0.4</td>
<td>0.35</td>
</tr>
<tr>
<td>S-2497</td>
<td>0.48</td>
<td>0.10</td>
<td>0.38</td>
<td>0.35</td>
</tr>
<tr>
<td>S-2421</td>
<td>0.97</td>
<td>0.62</td>
<td>0.35</td>
<td>0.35</td>
</tr>
</tbody>
</table>

Fig. 2. Reaction schemes for the amidolytic decomposition of the specific substrates by proteases.

Fig. 3. Cyclic voltammograms of the substrates at the carbon paste electrode (scan rate: 0.2 V/sec; in tris/NaCl/Gly/DMSO solutions at pH 7.45).
function \( \frac{I_{p/2}}{C} \), however, reflects the additive influences of the DMSO and glycine content. A calibration curve must therefore be assessed prior to the titration. In the following, the results will be referred to 5% v/v DMSO and 0.05M glycine.

**Influence of thrombin.**—Similarly, the influence of the thrombin content is studied through the modification of the anodic half-cycle of pADA oxidation. Results are presented in Table III for pH 7.46 and 8.47.

Proteins, especially thrombin (9) and albumin, are known to readily adsorb on synthetic surfaces. The anodic shift shown by the peaks in proportion to the added amount of protein probably accounts for that phenomenon, in the same way as glycine does. In the activity range in which the electrochemical assay is monitored, ca. 0.1 NIH unit, the disturbance caused by thrombin can be neglected. The electrochemical characteristics of the voltammograms are not drastically modified by the adsorption of proteins, provided that the concentrations fall below a given limit (Table III). For example, the reversibility of the charge transfer rate depicted by the \( E_{p/2} - E_{p} \) (1/2) values is maintained in aqueous solutions along the range for the activity determination of thrombin. Although the \( i_{p}/i_{pa} \) ratio offers no analytical exploitation, it can be remarked that it does not vary past a given amount of thrombin. This should be relevant for a protein coverage of the electrode surface reaching saturation. After its occurrence, it will likely slow down the chemical reversibility of the cycle, without affecting the electrochemical kinetic.

**Human Plasma and Whole Blood Dilutions**

**Study with the carbon paste electrode.**—Results are presented in Fig. 4 and 5 and Table III.

The comparison with voltammogram a of Fig. 4 traced at the reference pH 7.45 (the enzymatic assays are actually conducted at the latter pH) leads to the following remarks:

The dependence of the peak potentials with the pH of the solution is illustrated by voltammograms f, e, and a in Fig. 4, traced in H2SO4 0.7N, pH 3.29 and 7.45, respectively. The cathodic shift with increasing pH is relevant to the thermodynamics.

The background voltammograms of blood solutions up to 50% dilutions show no evolution electrochemically detectable up to at least 30 min. At the latter concentration, the anodic width of potential reaches 0.4V vs. SCE. This indicates that the protein content remains electrochemically inactive at the carbon paste electrode, at least with respect to charge transfer reactions.

It is worthy to note that as much as a 10% plasma or blood content reduces the peak current function \( \frac{i_{p/2}}{C} \) by less than 10%, see Table III and voltammograms b, c, and d in Fig. 4. This result is of great interest, keeping in mind that the antithrombin III (or thrombin) content usually estimated this way is included in few microliters of pure sample; therefore, there is no need to set up accurate calibration curves with respect to the final amount of proteins, prior to the assay.

Lowering the dilution of blood or plasma still maintains linear relationships in the amperometric determination of pADA. Figure 5 gives the \( \frac{i_{p/2}}{C} \) values as a function of the plasma or blood dilution. Once the data are corrected by taking into account the viscosity changes, there is no significant difference between these biological fluids. In particular, the presence of a heterogeneous phase brought by the figurative elements of blood (the haematocrit is about 45%) does not affect the applicability of the method. When undiluted blood or plasma samples are tested, it is just necessary to adjust the calibration curve to the conditions for the actual assay. This feature definitely marks the advantage of the electrochemical assay over the photometric one for trypsin-like enzymes in heterogeneous conditions.

### Table III. Electroanalytical data from the cyclic voltammograms of pADA solutions in the presence of thrombin, plasma, and blood dilutions.

<table>
<thead>
<tr>
<th>pH</th>
<th>( E_{pa} ) (V)</th>
<th>( E_{p/2} - E_{pa} ) (V)</th>
<th>( E_{p} - E_{p/2} ) (1/2) (V)</th>
<th>( \frac{i_{p/2}}{C} ) (A/L/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 8.47</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 NIH-U/ml</td>
<td>0.070</td>
<td>0.210</td>
<td>0.059</td>
<td>0.74</td>
</tr>
<tr>
<td>1 NIH-U/ml</td>
<td>0.069</td>
<td>0.209</td>
<td>0.058</td>
<td>0.75</td>
</tr>
<tr>
<td>pH 7.45</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 NIH-U/ml</td>
<td>0.100</td>
<td>0.060</td>
<td>0.049</td>
<td>0.74</td>
</tr>
<tr>
<td>0.1 NIH-U/ml</td>
<td>0.100</td>
<td>0.070</td>
<td>0.050</td>
<td>0.74</td>
</tr>
<tr>
<td>1 NIH-U/ml</td>
<td>0.100</td>
<td>0.100</td>
<td>0.050</td>
<td>0.74</td>
</tr>
<tr>
<td>Plasma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0%</td>
<td>0.100</td>
<td>0.040</td>
<td>0.070</td>
<td>0.77</td>
</tr>
<tr>
<td>10%</td>
<td>0.200</td>
<td>0.060</td>
<td>0.070</td>
<td>0.77</td>
</tr>
<tr>
<td>20%</td>
<td>0.220</td>
<td>0.070</td>
<td>0.070</td>
<td>0.77</td>
</tr>
<tr>
<td>40%</td>
<td>0.230</td>
<td>0.090</td>
<td>0.070</td>
<td>0.77</td>
</tr>
<tr>
<td>100%</td>
<td>0.290</td>
<td>0.110</td>
<td>0.070</td>
<td>0.77</td>
</tr>
<tr>
<td>Blood</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0%</td>
<td>0.100</td>
<td>0.040</td>
<td>0.070</td>
<td>0.77</td>
</tr>
<tr>
<td>10%</td>
<td>0.230</td>
<td>0.060</td>
<td>0.070</td>
<td>0.77</td>
</tr>
<tr>
<td>20%</td>
<td>0.220</td>
<td>0.080</td>
<td>0.070</td>
<td>0.77</td>
</tr>
<tr>
<td>100%</td>
<td>0.280</td>
<td>0.080</td>
<td>0.070</td>
<td>0.77</td>
</tr>
</tbody>
</table>

* Without glycine and without DMSO.
** With 5% DMSO.
† With 5% DMSO and glycine 0.02M.
Concentration of the depolarizer 0.4 mM. Mean deviation ±5 mV.
fluids such as blood, without turning to preliminary centrifugation.

In concentrated fluids and immediately following the mixing, the peak amplitude for pADA oxidation decreases up to ca. 50% of the original amplitude. Because the decrease is in relation to the dilution of the fluid and not to the analytical concentration of pADA, this trend cannot be attributable to macrophagic activity. It more probably concerns a modification of the electrode surface. This point will be discussed further, in light of the results obtained with the platinum electrode. The data presented here account for voltammograms traced passed this transient evolution.

Study with the smooth platinum disk electrode.—For practical purposes, it is obvious that solid electrodes potentially provide a very useful tool, easily applicable to automated or semi-automated analytical titrations, provided that several requisites are fulfilled:

1. The active surface of the electrode shall be sensitive enough and show evidence for reproducible peak currents, including constant background currents.

2. To attain this purpose, it may be essential to elaborate a precise protocol of pretreatment.

3. The amperometric titration shall be effectuated as frequently as possible during the course of the amido-lytic hydrolysis of the substrate. Thus, the same electrode surface shall operate several times while the potential is multicycled, without showing hysteresis, if the weakening of the analytic properties and without restriction of its effective area (with respect to adsorbed species, for example).

The following results have been obtained in aqueous or biological solutions buffered at pH 7.45. Blood and plasma dilutions were prepared just by mixing the biological fluids with the aqueous buffer.

Pretreatment procedure.—The background trace of metallic electrodes in aqueous solutions is quite sensitive to the chemical state of the surface and to adsorbed oxides. Therefore, the electrodes are often subjected to rather cumbersome prepolarizations, destined to obtain reproducible background voltammograms of the solvent. Inasmuch as the potential region of the amine undergoing oxidation is restricted to a few hundred millivolts, it would be suitable to apply a pretreatment procedure inside of this span. Results show that an interrupted triangular sweep of potential between −0.1 and +0.4V promotes the rise of a steady-state background cycle, whatever solvent is used, aqueous or biological. In the latter case, the duration of the pretreatment is even shortened. The integrated currents of the limiting voltammogram is always smaller than the initial trace. It is assumed that the gradual charging of the double layer is responsible for the decrease. After the application of the described prepolarization, the background currents superpose quite reproducibly, within a 10⁻² µA dispersion. In particular, no current increase or decrease develops on prolonged cycling over a period of time of at least 30 min. Here, the narrowness of the explored potentials and the gradual abation of the oxide peaks avoid some time-dependent effects related to the presence of proteins in the solution (9).

Refinements for the detection of pADA.—The optimum anodic and cathodic limits for the triangular sweep were analyzed in the light of the shape of the anodic curve. Results showed that an interrupted triangular sweep of potential between −0.1 and +0.4V promotes the rise of a steady-state background cycle, whatever solvent is used, aqueous or biological. In the latter case, the duration of the pretreatment is even shortened. The integrated currents of the limiting voltammogram is always smaller than the initial trace. It is assumed that the gradual charging of the double layer is responsible for the decrease.

Electrocatalytically speaking toward aromatic hydrocarbons or amines, platinum surfaces are known to behave poorly, in any event less than the carbon paste material. It is therefore not surprising that the pADA voltammograms show irreversible profiles. Although the criteria for electrochemical reversibility are far from being met, the amperometric detection gives nevertheless straight and proportional i_amps/C - pADA relationships, in any point comparable to those obtained using the carbon paste electrode. The amplitudes are in correspondence with the respective geometrical areas (Fig. 5).

Calibration of the platinum electrode in plasma and blood dilutions.—As was observed with the carbon paste electrode, the peak amplitudes recorded with the platinum electrode show little weakening in blood and plasma dilutions (voltammograms b and c, Fig. 6) up to 10%, if compared against a voltammogram on the reference (voltammogram a) in aqueous medium. It is also typical to note that the peak separation E_{pA} - E_{pB}
Fig. 6. Cyclic voltammograms of pADA at the pretreated smooth platinum electrode. (Scan rate 0.2 V/sec; tris/NaCl/Gly/DMSO pH 7.45). a, Aqueous solution; b, 1/10 diluted whole blood; c, 1/10 diluted plasma; d, undiluted whole blood.

decreases with the biological mixtures. Probably, the occurrence of the coated proteins favors the chemical reversibility of the redox couple by slowing down the propensity to migrate away (as might occur with more effective adsorption in the presence of coated proteins), although without interfering with the diffusion rate toward the electrode surface.

The more interesting result concerns the peak height for pADA oxidation which fairly well compares in aqueous buffers with even pure blood (voltammogram d in Fig. 6). These findings give rise to the data plotted in Fig. 5 for the platinum electrode in correspondence with those referring to the carbon paste electrode. The comparison is quite attractive inasmuch as it shows the neglectable influence of the blood or plasma content of the solution onto the calibration. In turn, these results indicate that the transient decrease of the peaks traced with the carbon paste electrode are to be related to surface effects, but not to the consumption of the depolarizer by the biological fluids.

It is obvious that the insensitivity to the biological environment confers to the pretreated platinum electrode a definite advantage over the other electrode systems. It also affords a substantial relief in the practical procedure preceding the enzymatic assay.

On the other hand, calibration curves proving that the measured signal is a linear function of concentration have been published in other papers of this series (3); the function is essentially identical for aqueous solutions, human plasma, and whole blood dilutions.

Conclusion

An electroanalytical method for the study of enzymatic kinetics is proposed. The use of modified electrode surfaces through the adsorption of proteins affords a new insight into the behavior of clotting enzymes in the presence of insoluble materials or into whole blood dilutions.

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