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# Heat Stable Langmuir–Blodgett Film of Glutathione-S-Transferase

Francesco Antolini,<sup>†</sup> Sergio Paddeu,<sup>‡</sup> and Claudio Nicolini<sup>\*,‡</sup>

*Institute of Biophysics, School of Medicine, University of Genoa, Via Giotto 2, 16153 Genoa Sestri Ponente, Italy, and Technobiochip, Via Roma 28, 57030 Marciana (LI), Italy*

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Glutathione-S-transferase (GST) is an enzyme able to conjugate glutathione to electrophilic molecules such as 1-chloro-2,4-dinitrobenzene or some triazinic pesticides, such as atrazine, with production of protons. The Langmuir–Blodgett (LB) technique was utilized and optimized to obtain stable monolayers of this enzyme. The film was then characterized by means of circular dichroism spectroscopy, nanogravimetry, and spectrophotometry in order to determine the structure, surface density, and functional activity of the enzyme when packed in the monolayers. The functional analysis of the LB film was also conducted as a function of the number of layers and of the temperature. While the kinetic response as a function of layers suggested that only the external layer was active, the film activity as a function of temperature indicated that, as shown earlier for other proteins, GST in a LB film appears to strictly preserve its functional activity and thus its structure up to 423 K. The enzymatic activity was finally tested in solution with the potentiometric alternating biosensor (PAB) system, with the minimum detectable amount of 1-chloro-2,4-dinitrobenzene being 25  $\mu$ M. The analysis was highly encouraging for a biosensor application, considering that both structure and function appear largely preserved after the deposition.

## Introduction

Protein film formation and deposition on a solid substrate make it possible to achieve a material with new properties such as thermal stability,<sup>1</sup> organization of the molecules in an ordered array useful for bioelectronic devices,<sup>2,3</sup> or simply covering the transducer with biomolecules to obtain a biosensor.<sup>4,5</sup> The Langmuir–Blodgett (LB) technique<sup>6–9</sup> is a method which permits the formation of monolayers of several types of biomolecules<sup>10</sup> at the air–water interface and their transfer onto a solid substrate. This technique shows a lot of advantages: first of all the film formation at the air–water interface is very well controlled; moreover the film organized in a two-dimensional array can be deposited onto a solid substrate and then characterized.

Our main interest was to realize enzyme LB films by direct spreading of the enzyme at the air–water interface and to deposit them onto a solid support in order to demonstrate that the activity of the enzyme in the film was preserved both at room temperature and after thermal treatment.<sup>1,11</sup>

In previous works it was shown that the protein activity in the LB film is still maintained,<sup>11–13</sup> despite the high

surface tension at the air–water interface. In particular this was shown for monolayers of membrane proteins<sup>1</sup> and antibodies,<sup>11</sup> obtained by directly spreading the protein solution at the air–water interface. The direct spreading of enzymes at the air–water interface and the corresponding film activity determination were first performed by Langmuir;<sup>14</sup> in more recent years<sup>15,16</sup> the enzymes were immobilized by absorption on preformed LB films at the air–water interface. The enzymatic reaction here analyzed was the conjugation of reduced glutathione (GSH) to electrophile substrates such as 1-chloro-2,4-dinitrobenzene (CDNB) catalyzed by glutathione-S-transferase (GST).<sup>17,18</sup> In mammalian and plant cells GST is an agent devoted to their detoxication:<sup>19–21</sup> in fact the product of the reaction is often soluble in water and, therefore, can be removed from the cell. The GST, that consists of two subunits with a total molecular weight of 45 000,<sup>17,18,22</sup> was chosen for two important reasons: (i) the reaction with the CDNB can be easily spectrophotometrically monitored and (ii) the GST action on electrophile herbicides (e.g. atrazine)<sup>23–25</sup> produces

<sup>†</sup> Technobiochip.

<sup>‡</sup> University of Genoa.

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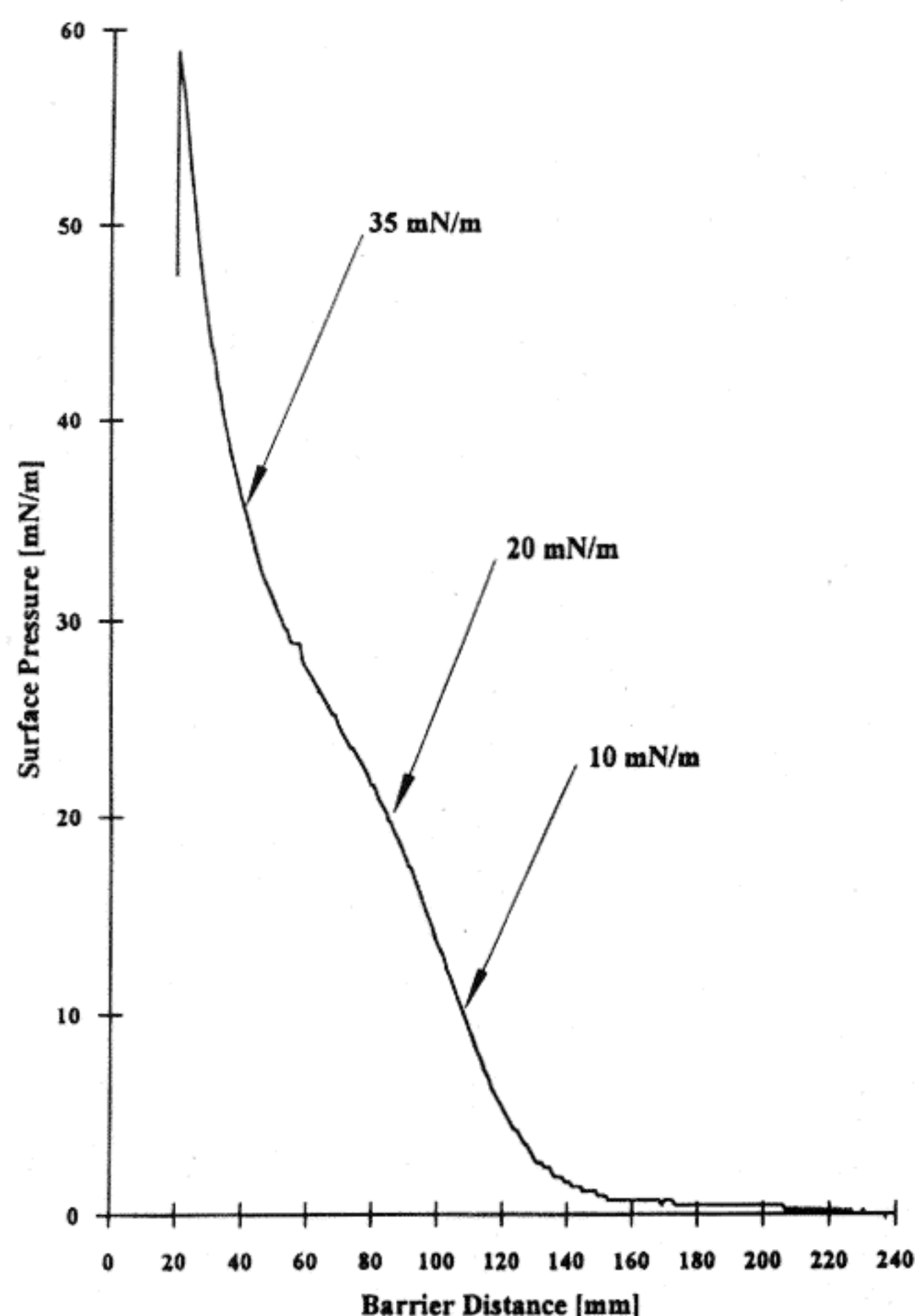
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**Figure 1.** Isotherm of GST. The arrow indicates the deposition surface pressure. The surface pressures of deposition for CD spectra are indicated.

protons detectable by means of the PAB system.<sup>26,27</sup> This is a new biosensing system based on a light addressable potentiometric sensor (LAPS)<sup>28</sup> allowing the monitoring of several kinds of phenomena, such as the cell viability<sup>29</sup> and the enzymatic activity.<sup>30,31</sup>

First of all, the deposited GST LB film was characterized by studying the protein secondary structure content in the film by means of circular dichroism and the area per molecule of the protein in the film by means of nanogravimetry. The enzymatic assay of the film was carried out both as a function of layers and as a function of thermal treatment at 353 and 423 K, in order to investigate the heat stability of the GST catalytic activity as shown elsewhere in the case of membrane proteins and antibody films.<sup>1,11</sup>

A preliminary work on the PAB system was also carried out in solution in order to estimate whether the enzymatic reaction was detectable in the presence of different substrate concentrations.

Since GST is able to catalyze the conjugation of GSH with electrophilic molecules, such as atrazine, this system can also be used as a pesticide biosensor.

## Materials and Methods

**Reagents and Equipment.** GST from equine liver (EC 2.5.1.18), GSH, and CDNB were purchased from Sigma Chemical Co. The water, with a resistivity greater than  $18 \text{ M}\Omega\cdot\text{cm}^{-1}$ , was purified by means of Milli Q water purification system. The stock solution of GSH was prepared at a final concentration of 250 mM and buffered at pH 6.5. The stock solution of CDNB was prepared by dissolving CDNB in EtOH till a final concentration of 50 mM. The silicon substrate used for the LB deposition was provided by IRST, Trento, Italy. The spectrophotometric analysis was carried out with a double beam spectrophotometer Jasco 7800. The circular dichroism spectra were measured with a Jasco 780 spectropolarimeter. The resonance frequency of the quartz resonator utilized for the nanogravimetric assays was about 10 MHz, and the equipment for the determination of the frequency shift was the same as reported in the literature.<sup>33</sup>

The PAB system for measuring the enzymatic activity was as described elsewhere.<sup>26,27</sup>

The PAB reaction chamber employed for monitoring the enzymatic activity was made in Teflon, with a maximum volumetric capacity of 1.2 mL. The LAPS silicon transducer (n type, sensitivity  $50 \text{ mV}\cdot\text{pH}^{-1}$ ; IRST, Trento, Italy) had a silicon nitride ( $\text{Si}_3\text{N}_4$ ) surface as the sensitive element, and therefore, it was able to reveal the  $\text{H}^+$  ions in solution.<sup>34</sup>

**Film Formation.** The surface properties of GST at the air-water interface were examined with a trough of  $240 \times 100 \text{ mm}^2$ , and the surface pressure was measured using a Wilhelmy balance having an accuracy of  $0.2 \text{ mN}\cdot\text{m}^{-1}$  (MDT Corp.). The subphase used was pure water. The protein was dissolved in a buffer solution of 1 mM phosphate, 1 mM EDTA, 50 mM NaCl at pH 6.5 to a final concentration of  $1 \text{ mg}\cdot\text{mL}^{-1}$ . After that,  $40 \mu\text{L}$  of GST solution was spread on the surface and immediately compressed by barriers with a speed of  $3 \text{ mm}\cdot\text{s}^{-1}$ . The isotherms were reproducible with an accuracy of 1%.

The surface pressure of deposition was in the range  $10\text{--}35 \text{ mN}\cdot\text{m}^{-1}$ . Silicon nitride ( $\text{Si}_3\text{N}_4$ ) wafer, silicon, and quartz, used as substrates, were cleaned as follows: the wafer was immersed in sulfochromic solution for 10–15 min, washed first with water, then with acetone, and finally with purified water; it was then dried with nitrogen flux. The samples were used immediately for the deposition according to the Langmuir–Schaefer technique (horizontal lifting).<sup>14</sup> The samples contained 1–30 monolayers depending on the type of measurement.

**Film Characterization.** (a) *Circular Dichroism Analysis.* The dichroic signal was obtained for GST protein in solution and in film. The buffer solution was the same used for film deposition, and the GST concentration was  $23.7 \mu\text{M}$ . The molar ellipticity ( $\Theta$ ) was evaluated as reported in ref. (1) considering that the molecular weight of the GST was 45 000,<sup>18</sup> the path of the cuvette was 0.01 cm and the number of amino acids of GST was 434.<sup>22</sup>

The sample of GST film for CD was prepared by depositing the enzyme on a quartz slide previously cleaned, at surface pressures of 10, 20, and  $35 \text{ mN}\cdot\text{m}^{-1}$ . Cleaning was carried out by immersing the quartz slide for 10–15 min in the sulfochromic solution and then washing it in purified water and drying with nitrogen flux. The molar ellipticity of the film was calculated using formulas reported in the literature.<sup>1</sup>

(b) *Nanogravimetric Analysis.* The deposition on the quartz resonator was done on both resonator sides, and after each covering, the resonator was dried with nitrogen flux and then the frequency shift was measured. The frequency value was taken 5 min after the connection of the quartz to the circuits in order to stabilize the measure. The Sauerbrey equation<sup>35</sup> correlates the frequency shift, with the mass shift as shown in eq 1, where  $f_0$ ,  $A$ ,  $\rho$ , and  $l$  are the frequency of the resonator

$$\frac{\Delta f}{f_0} = - \frac{\Delta m}{A \rho l} \quad (1)$$

without any covering, the covered area, the density of the quartz, and the thickness of the resonator, respectively. All these values

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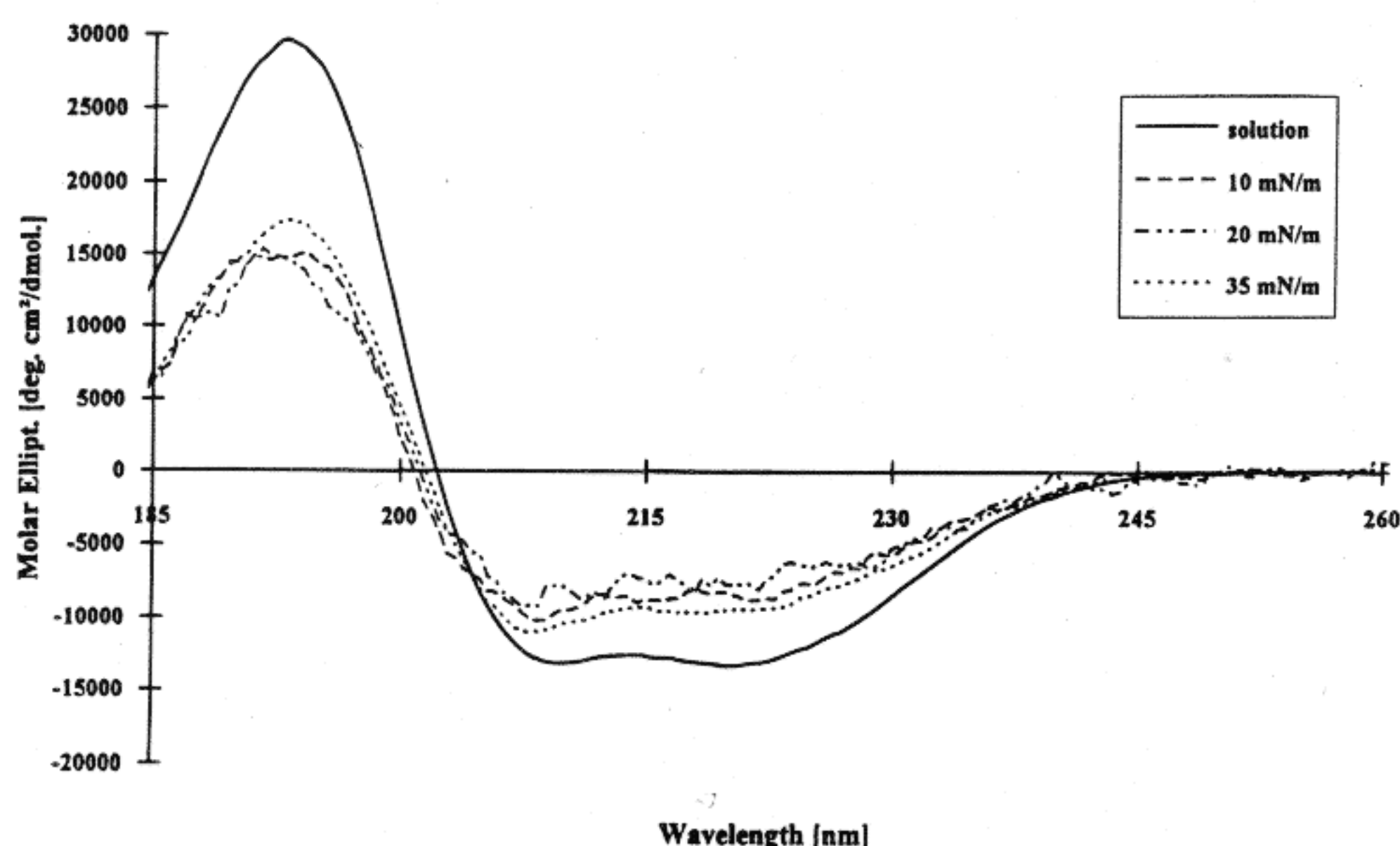
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**Figure 2.** CD spectra of GST in solution (solid line) and in films at three different pressures (dashed lines). The spectra are recorded between 185 and 260 nm.

**Table 1.** Percentage Values of  $\alpha$ -Helix,  $\beta$ -Sheet,  $\beta$ -Turn, and Random Coil of the GST Molecules in Solution versus a LB Film Obtained from CD Spectra by Means of a Modified HJ Procedure<sup>a</sup>

condition	$\alpha$ -helix	$\beta$ -sheet	$\beta$ -turn	random coil
soln	43	17	20	20
film	22 $\pm$ 1	24 $\pm$ 1	25 $\pm$ 1	28 $\pm$ 2

<sup>a</sup> Deposition pressure of 20 mN·m<sup>-1</sup>.

are constant and therefore eq 1 can be written as follows:

$$\Delta s = -K\Delta f \quad (2)$$

where  $\Delta s = \Delta m/A$  represents the surface density shift,  $\Delta f$  is the frequency shift, and  $K$  is a constant depending upon the physical parameter of the utilized resonator. The  $K$  value was estimated according to the procedure reported elsewhere<sup>33</sup> to be 0.0295 ng·Hz<sup>-1</sup>·mm<sup>-2</sup>. Ten depositions were carried out on the resonator, and the frequency shift was measured after each deposition.

**Enzymatic Activity of GST in Solution and in a LB Film.** The enzymatic activity was tested both in solution and in a LB film. The kinetics of the product formation (1-glutathione-2,4-dinitrobenzene (GDNB)) was followed with the spectrophotometric assay at 340 nm with a cuvette of 1 cm optical path length and a volume of 1 mL. All the kinetic experiments were carried out at 298 K and with the reference cuvette containing all the reagents except the enzyme. The  $\Delta\epsilon$  of the reaction and the  $\epsilon_{\text{GDNB}}$  (molar extinction coefficient) at 340 nm are 9.6<sup>18</sup> and 0.49 mM<sup>-1</sup>·cm<sup>-1</sup>, respectively. The buffer used was 1 mM phosphate, 1 mM EDTA at pH 6.5.<sup>17</sup> The effect of the ionic strength on the enzymatic activity was evaluated by varying the salt concentration from 0 to 100 mM NaCl. These very low buffer capacity and high salt conditions were used as they are the best conditions for PAB applications.<sup>27</sup> The kinetic parameters,  $K_m$  and  $V_{\text{max}}$ , of the GST reaction were determined in such conditions by means of the Lineweaver-Burk equation (data not shown).

The analysis of the LB film activity was done by immersing the sample in a 3 mL cuvette containing buffer solution with 0.5 mM CDNB and GSH at 2.5 mM and a final volume of 2 mL. The activity of the enzyme film was obtained by monitoring the kinetics for 1000 s and evaluating the slope. This procedure was followed for the determination of the activity by varying the number of layers in the film. All these measurements were carried out under continuous stirring using a magnetic microstirrer (Bioblok Scientific) at a speed of 600 rpm.

The activity of the LB films was also tested after dry thermal treatment of the samples at different temperatures. The deposited film was heated at the desired temperature for 10 min, and then it was kept at room temperature for 5 min. After this treatment the enzymatic activity was monitored as previously described.

**PAB System: Monitoring of Enzymatic Activity in Solution.** The scheme of the PAB system is illustrated in detail elsewhere.<sup>27</sup> The output signal of the LAPS transducer is a photocurrent<sup>28</sup> which is converted to a voltage (V). A pH variation, which takes place in the reaction chamber, is monitored by following the changes of this voltage directly, as shown below in eq 3 where  $\Delta V$  is the output voltage variation,  $\sigma$  is the sensitivity

$$\Delta \text{pH} = \frac{\Delta V}{\sigma \alpha} \quad (3)$$

of the silicon transducer, and  $\alpha$  is the maximum slope of the output characteristic curve.<sup>30,36</sup>

Utilizing the static configuration of this system,<sup>27</sup> the pH variation due to the enzymatic reaction was measured. A 1 mL aliquot of enzymatic mixture was prepared and immediately injected into the reaction chamber. The output signal was monitored for 20 min.

The pH at the equilibrium state of the enzymatic reaction was calculated by extrapolating the tendency of the exponential fit at infinite time, as shown in eqs 4 and 5, where  $\text{pH}_t$  is the pH

$$\text{pH}_t = \text{pH}_\infty + \overline{\Delta \text{pH}} e^{-V_0 t} \quad (4)$$

$$\overline{\Delta \text{pH}} = \text{pH}_0 - \text{pH}_\infty \quad (5)$$

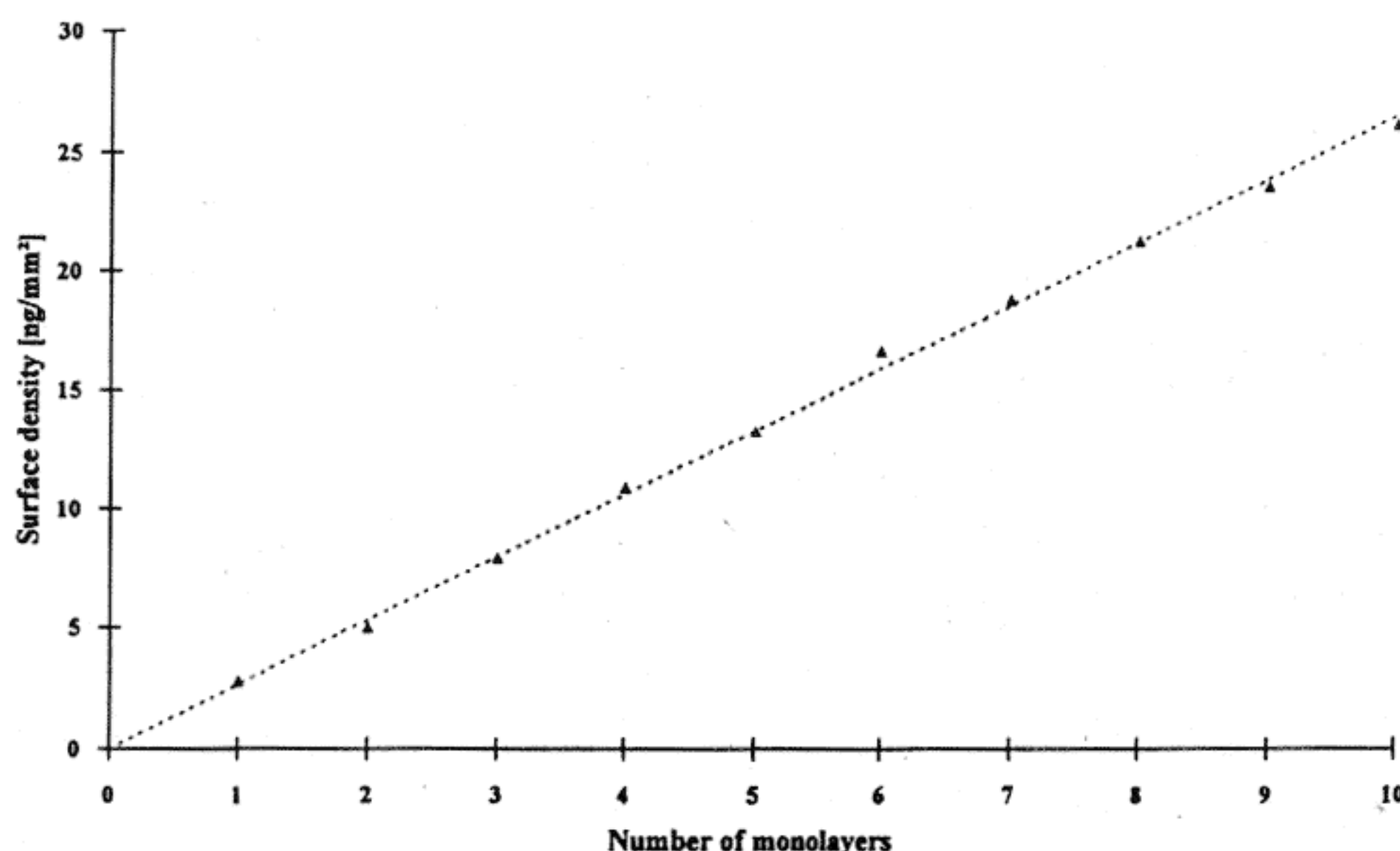
value at time  $t$ ,  $\text{pH}_\infty$  is the equilibrium pH,  $\text{pH}_0$  is the pH at time 0, and  $V_0$  is the initial speed of the enzymatic reaction. The  $\overline{\Delta \text{pH}}$  was calculated for each utilized CDNB concentration. Eq 4 allows us to fit the experimental data and therefore to correlate the kinetic reaction to the pH variation; the rate of pH variation is equal to  $V_0$  and corresponds to the slope of the output signal.<sup>30</sup>

## Results and Discussion

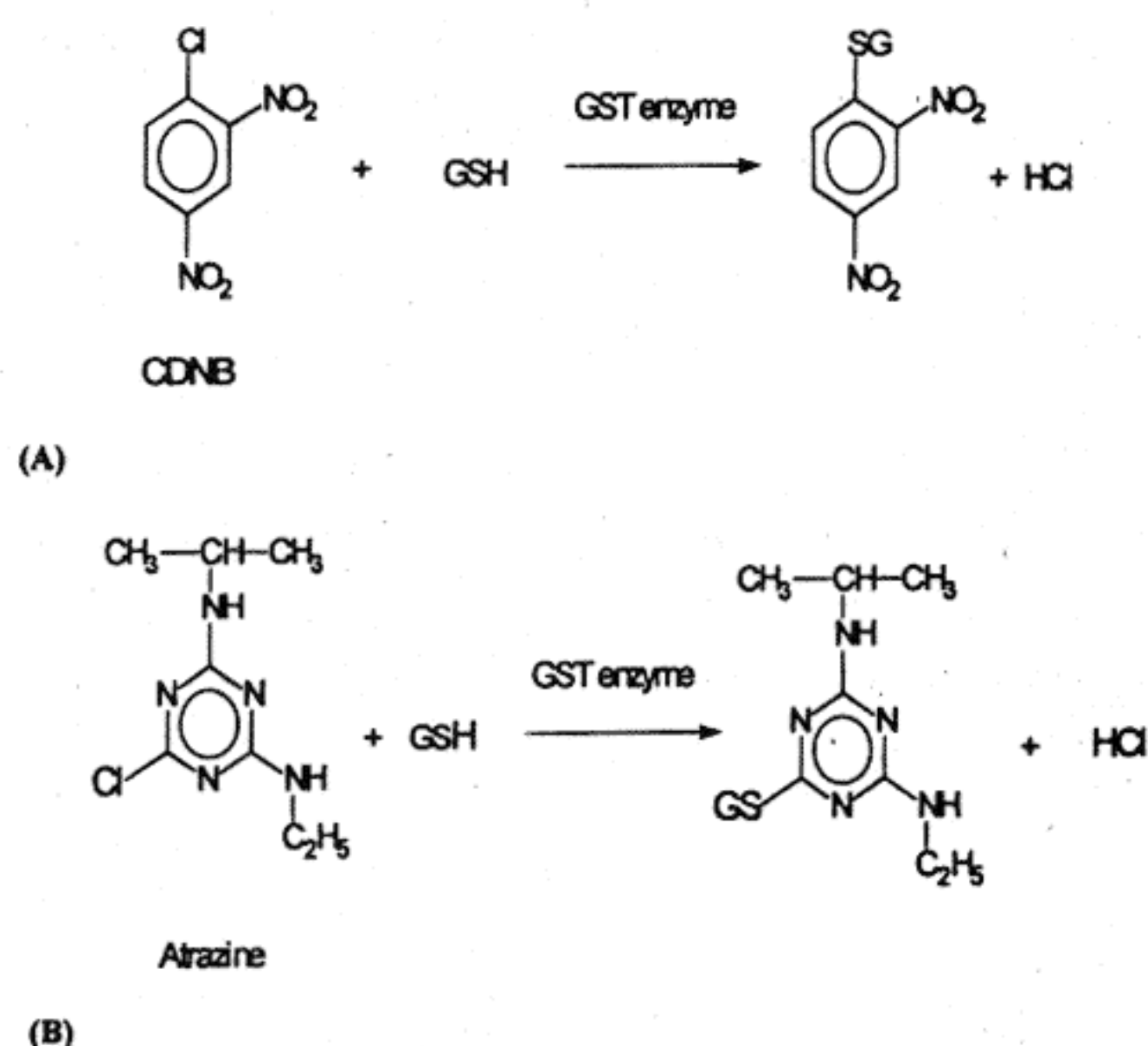
**Film Characterization.** The isotherm shown in Figure 1 indicates the presence of the film at the air-water interface and three slope changes. The high speed of compression was used in order to decrease the time during which the proteins are unpacked at the air-water interface. In this condition, the surface tension of water can denature the protein itself.<sup>11</sup> The deposition pressure was chosen by considering the isotherm (Figure 1) and the CD spectra (Figure 2) of the deposited film at 10, 20, and 35 mN·m<sup>-1</sup>. The film was deposited with the Langmuir-Schaefer (horizontal lifting) technique<sup>14</sup> in order to obtain a good deposition ratio.<sup>10</sup> The CD signal,

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**Figure 3.** Nanogravimetric assay; the points represent the experimental mass shift after each deposition. The line is the resulting regression curve with a slope equal to  $2.62 \text{ ng}\cdot\text{mm}^{-2}$ .



**Figure 4.** Conjugation of CDNB (A) and atrazine (B) with GSH catalyzed by GST.

for all the pressures, was practically equal, indicating that no great differences were present in the protein secondary structure.

At the pressure of  $35 \text{ mN}\cdot\text{m}^{-1}$  the film at the air–water interface was unstable and the film instability at the air–water interface (defined as the percentage decrease of film area with respect to its initial value) at this deposition pressure, versus time, was about 15%/min. This, instead, points to a likely film collapse and to protein sinking into the subphase. At the deposition pressure of 10 and  $20 \text{ mN}\cdot\text{m}^{-1}$  the GST LB film was formed and stable (instability less than 2%/min). Moreover, at the pressure of  $20 \text{ mN}\cdot\text{m}^{-1}$  the GST molecules in the film were more packed than at  $10 \text{ mN}\cdot\text{m}^{-1}$  (as apparent by nanogravimetry), improving the quality of the LB film.

The CD spectra gave additional information about the secondary structure of the protein in the film and can be used as the first indication for the film activity. Figure 2 represents the CD spectra of both solution and film ( $20 \text{ mN}\cdot\text{m}^{-1}$ ) at room temperature. The values of  $\alpha$ -helix,  $\beta$ -sheet,  $\beta$ -turn, and random coil percentage of GST, calculated by a modified HJ procedure,<sup>37</sup> are also indicated in Table 1. The results show that the protein, when arranged in a LB film, lost part of its  $\alpha$ -helix structure

and increased the random coil content. This can be due to the partial denaturation of the molecule during spreading at the air–water interface or during deposition, possibly due to the high surface tension of water. Part of these changes can be derived from the reorganization which the protein undergoes when it is closely packed in a LB film. However, the structure was not completely lost and this suggested that the GST enzymatic activity could be partially retained.

Preliminary X-ray scattering data of 30 layers GST film gave no appreciable peaks, indicating that the enzymatic LB film was amorphous and the surface was not homogeneous (manuscript in preparation).

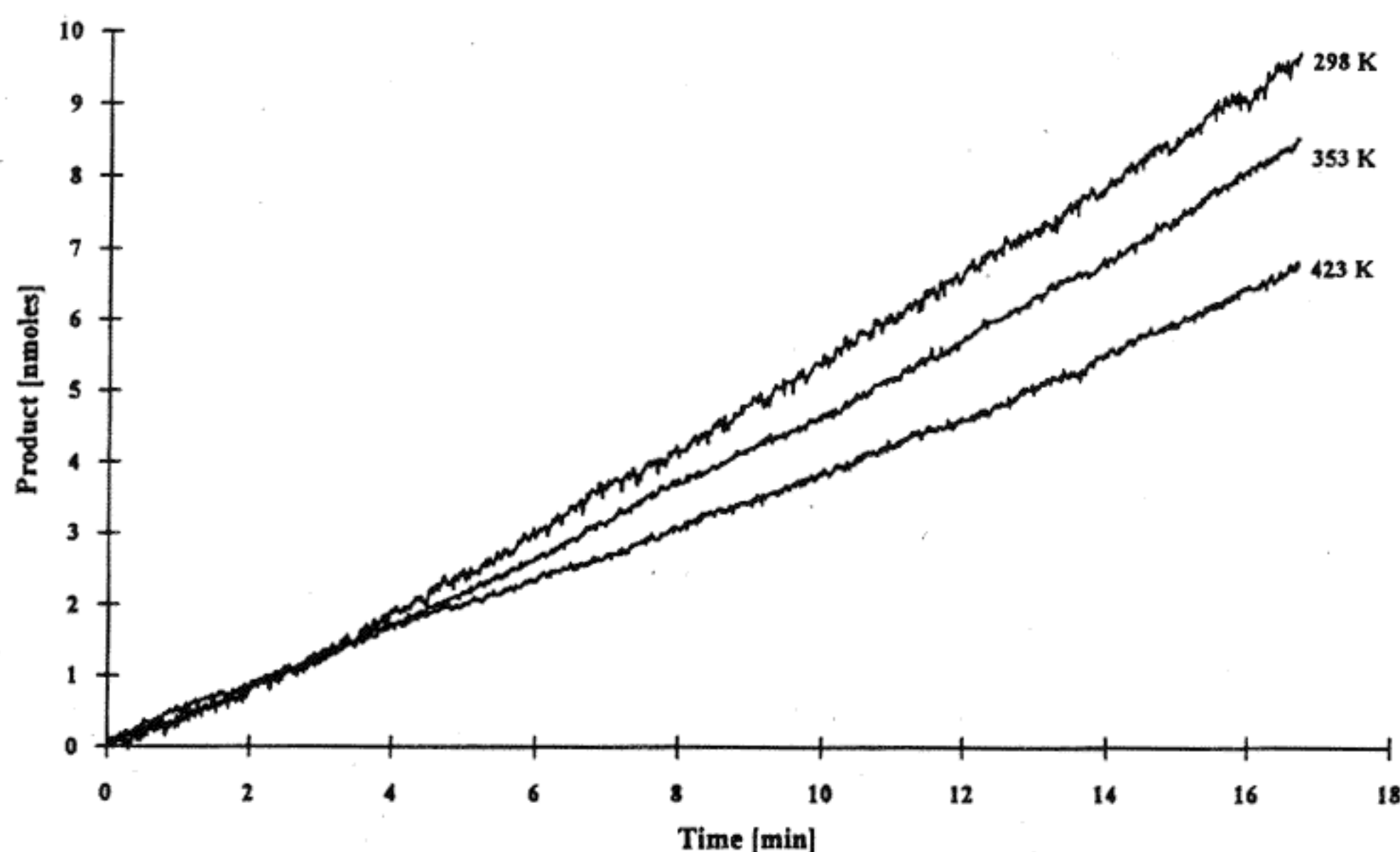
The surface density and structure of the film were tested with nanogravimetry. Nanogravimetry allows us to determine the mass of protein films after each deposition and, therefore, to evaluate the reproducibility of the deposition, the total amount of deposited protein, the surface density, and the area per molecule of GST in the film. The nanogravimetric assay showed that the depositions were reliable with a percentage error of about 30% (Figure 3). The surface density showed also that the amount of protein deposited on the substrate after each deposition was  $2.62 \pm 0.87 \text{ ng}\cdot\text{mm}^{-2}$  (equivalent to  $5.82 \times 10^{-2} \pm 1.93 \times 10^{-2} \text{ pmol}\cdot\text{mm}^{-2}$ ) of pure protein. The purity of the deposited protein was due to the fact that LB film formation allows for elimination of soluble contaminants (salts present in lyophilized enzyme) in water, during the spreading of the protein solution at the air–water interface. The area per molecule occupied by a GST molecule can be estimated by calculating the surface density. The observed average area per molecule was  $2800 \pm 700 \text{ \AA}^2$ , which was slightly less than the area per molecule calculated from crystallographic data,<sup>22</sup> being about  $3242 \text{ \AA}^2$ , which was obtained by taking into account the random orientation of the GST having the shape of a parallelepiped,  $53 \times 62 \times 56 \text{ \AA}$ . The result indicates that the proteins in the film are closely packed because the area per molecule is slightly less than that expected theoretically, in agreement with the slight secondary structural changes observed by CD spectra.

**Enzymatic Activity Assay.** The enzymatic activity of the film was checked with CDNB as a standard substrate

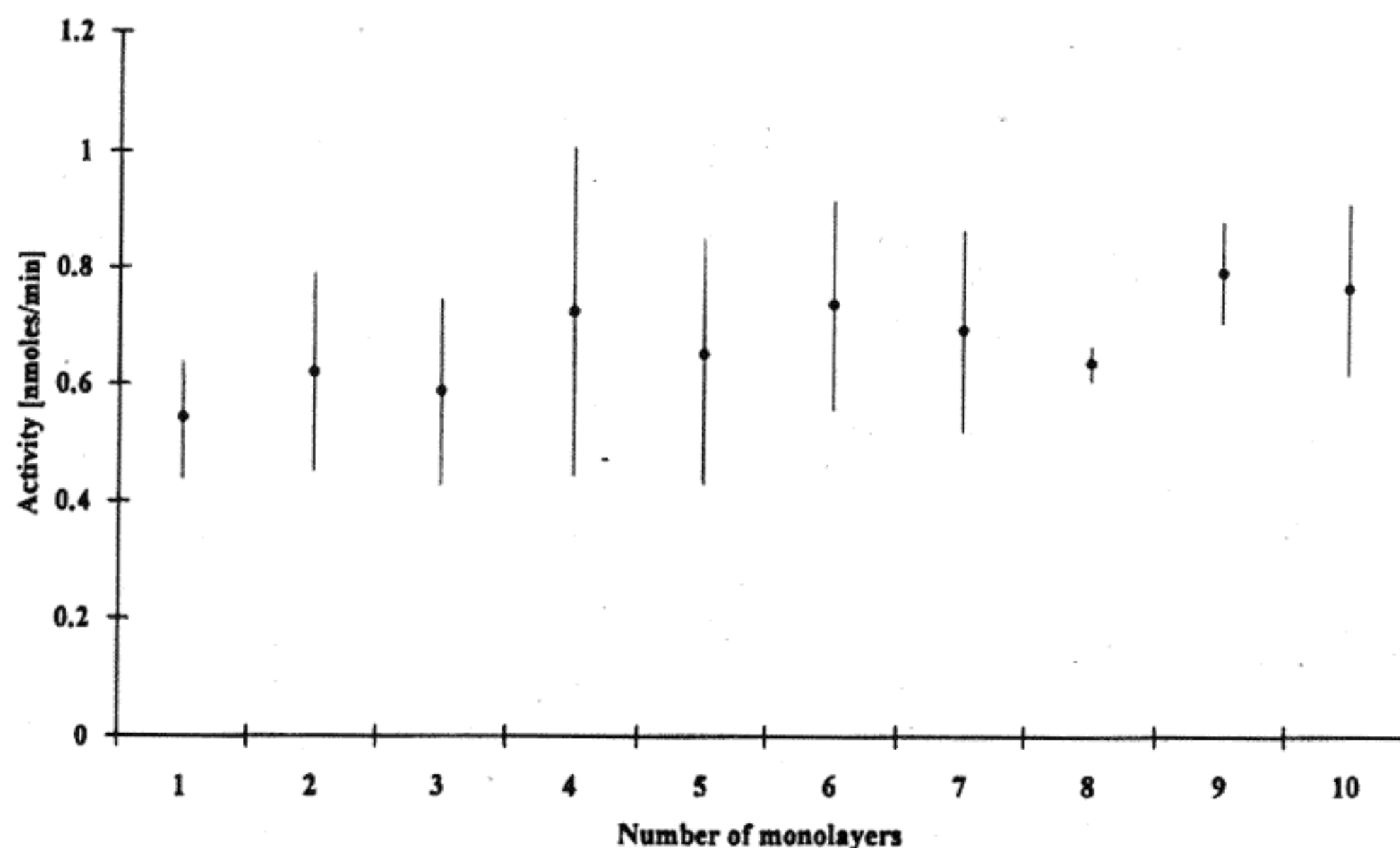
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**Figure 5.** Kinetic curve of the enzymatic reaction when GST is deposited onto silicon substrate by utilizing the LB technique at three different temperatures. The film is 1 one layer thick. The produced GDNB is determined versus time at room temperature for 2.5 mM GSH, 1 mM phosphate buffer, 1 mM EDTA, NaCl 50 mM at pH 6.5 in a volume of 2 mL in the presence of stirring (above).



**Figure 6.** Dependence of activity on the number of monolayers. For each point is given the error (for a confidence level of 95%). The reaction volume is 2 mL.

**Table 2. Specific Enzymatic Activities of the GST Enzyme Molecules as a Function of Organization (Free in Solution, Spread, and LB Film) and Temperature<sup>a</sup>**

temp (K)	soln <sup>b</sup>	spread <sup>b</sup>	LB film <sup>b,c</sup>
298	$5.83 \times 10^{11}$	$2.8 \times 10^{10}$	$9.9 \times 10 \pm 2.9 \times 10^{10}$
353	0	$2.1 \times 10^{10}$	$8.2 \times 10 \pm 2.4 \times 10^{10}$
423		$1.6 \times 10^{10}$	$6.3 \times 10 \pm 1.9 \times 10^{10}$

<sup>a</sup> The activities comparison shows that the dehydration prevents the protein denaturation. In fact, at 353 K the thermal treatment inactivates the enzyme in solution completely, while the dry enzyme maintains its catalytic function up to 423 K. <sup>b</sup> Specific enzymatic activities expressed in  $\text{nmol} \cdot \text{min}^{-1}$  per mole of enzyme. <sup>c</sup> The LB film is formed by one layer.

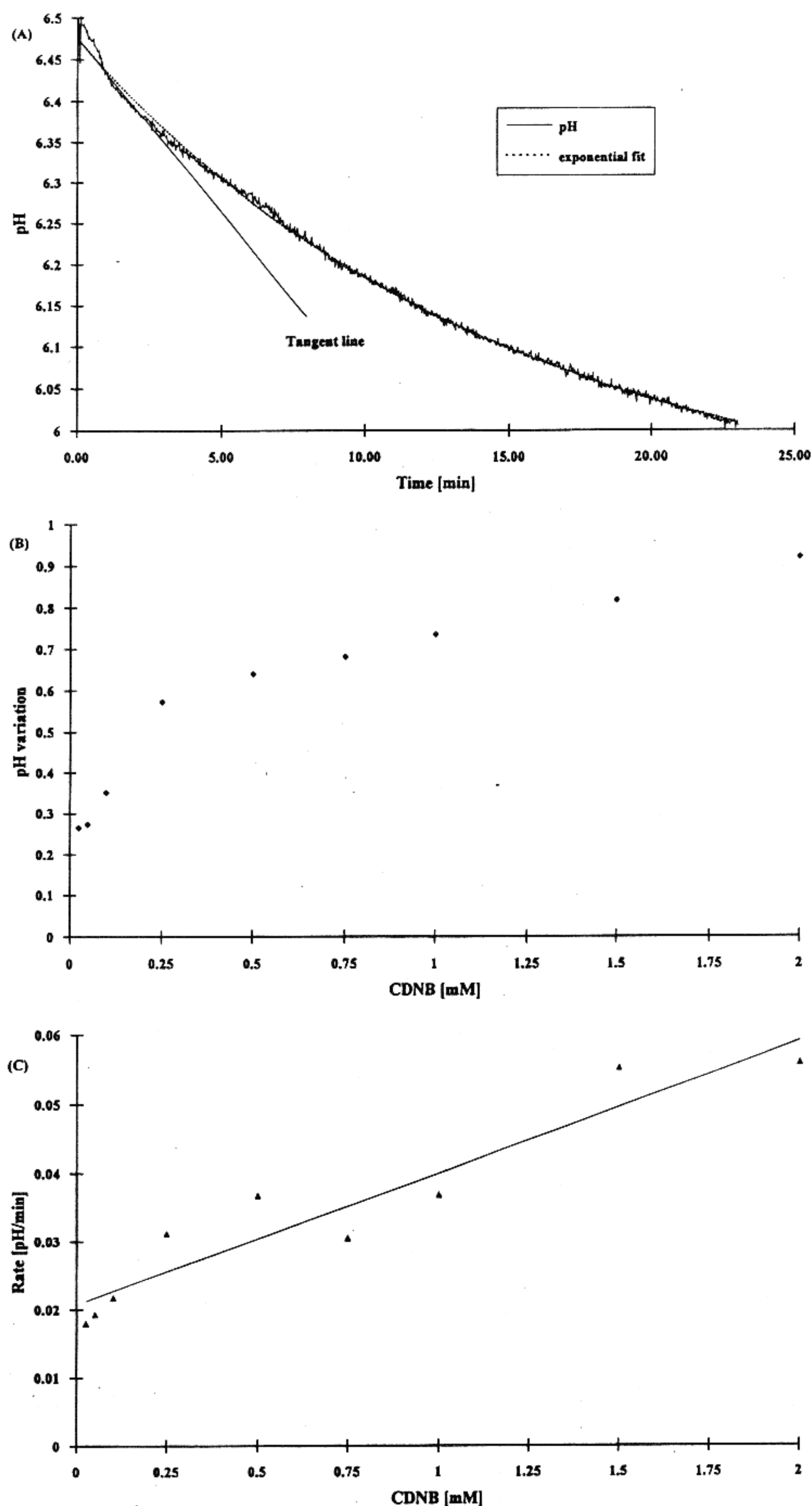
(Figure 4A). For the biosensing application this system is very interesting because GST is able to modify a class of electrophile pesticides such as atrazine (Figure 4B). To apply the GST film to the PAB system, it is necessary to decrease the buffer capacity<sup>30</sup> and increase the solution ionic strength usually utilized for the standard enzymatic assay. The buffer concentration was chosen equal to 1 mM, in order to minimize the buffer capacity. The effect of ionic strength indicates that there is practically no

**Table 3. Percentage Activities of the GST Enzyme Organized as a Spread (Random Orientation) and LB Film Compared to That in Solution<sup>a</sup>**

temp (K)	soln	spread	LB film <sup>b</sup>
298	100	4.8	17.0
353		3.6	14.0
423		2.7	10.8

<sup>a</sup> The effect of packing induced by the LB technique with respect to the random orientation is underlined by the enhanced thermal stability. <sup>b</sup> The activity in the LB film was determined for one layer.

influence of salt in the enzymatic activity of GST (data not shown). On the basis of these results the next experiments were carried out with a buffer at pH 6.5<sup>17</sup> with 50 mM NaCl added in order to achieve a good output signal from the PAB system. The kinetic parameters of the GST enzyme in these new conditions were also obtained by means of the Lineweaver–Burk curve. The values of  $V_{\text{max}}$  and  $K_m$  are  $700 \text{ mol} \cdot \text{min}^{-1}$  per mole of enzyme and 0.13 mM, respectively. Despite the new measurement conditions, the orders of magnitude of these values were in agreement with those of the literature.<sup>18</sup>



**Figure 7.** (A) Acidification curve (solid line) due to the enzymatic reaction monitored with PAB and exponential fitting (dashed line). The concentrations of GST, CDNB, and GSH were 0.22  $\mu$ M, 1 mM, and 2.5 mM, respectively. (B)  $\Delta$ pH variation (absolute value), referred to infinite time, and (C) rate of pH variation (absolute value) versus CDNB concentration. The concentrations of the enzyme and of the GSH in the PAB chamber are 0.22  $\mu$ M and 2.5 mM, respectively. The CDNB concentrations are 0.025, 0.05, 0.1, 0.25, 0.5, 0.75, 1, 1.5 and 2 mM. The pH decreases starting from 6.5.

**Film Enzymatic Activity.** The kinetic data on the GST monolayer are shown in Figure 5. The kinetic curve

was reproducible within experimental error due to the deposition process (see nanogravimetry data). The film



activity was also studied as a function of the numbers of deposited monolayers, in order to verify whether the film was totally or partially active.

The activity as a function of layers (Figure 6) indicates that mostly the upper layer is active, suggesting that a quite limited diffusion process takes place inside the film when more than one monolayer is deposited. This fact can be due to the packing of the molecules, that prevent the access of the reagents to the lower layers and/or the partial inactivation of the lower layers by the enzyme molecules of the upper one, which inhibits the active sites of the protein.

In Tables 2 and 3 the specific and the percentage activities of the LB film with respect to the solution are presented. The activity of the LB film with respect to the solution is lower, suggesting that there is a partial inactivation of GST in the film caused by either the partial denaturation and/or the close packing.

Conversely, while in solution the thermal treatment completely inactivates the enzyme and destroys its structures already at 353 K, in the LB film the enzymatic activity is still maintained up to 423 K, with only a slight decrease in the functional activity (Figure 5, Tables 2 and 3) and secondary structure (data not shown).

The enzymatic activity decrease was of about 18% at 353 K and 37% at 423 K with respect to that at room temperature (Table 3 and Figure 5). The maintenance of activity after thermal treatment agrees with previous data,<sup>11</sup> suggesting that the thermal stability induced by our optimization of the LB procedure is a general behavior of all protein classes. The effect of the order induced by LB organization in respect to a random oriented protein film (spread film) was also proved (Table 3), considering the fact that the inactivation was fairly pronounced, 25% at 353 K and 44% at 423 K, respectively.

The thermal stability phenomenon can be caused mainly by the close packing of the protein in the LB film and by the film dehydration,<sup>1,11</sup> which prevent the protein denaturation. On the contrary, in solution, already at 353 K the enzymatic activity is practically absent (Table 2 and 3).

**PAB Application.** The enzymatic reaction in solution was tested with the PAB system<sup>27</sup> as a first step for future biosensing applications. Using the first order kinetic law (eq 4), the pH value at the equilibrium state can be correlated with the substrate concentration and the fit of the experimental measure is obtained (Figure 7A). The theoretical curve was used to find the pH value at the equilibrium state (Figure 7B) and to obtain the rate of pH variation (Figure 7C) for each considered CDNB concentration. These quantities were correlated to the substrate concentrations to make a calibration curve of the sensor.

A linear correlation is apparent between  $\Delta\text{pH}$  and CDNB in the range 0.25–2 mM (Figure 7B) and between the pH rate variation and CDNB in the range 0.025–2 mM (Figure 7C).

As shown earlier with a different enzyme under a similar configuration,<sup>30</sup> the problems related to the diffusion process apparent at low CDNB concentrations in the PAB static reaction chamber (utilized in this experiment carried out with the enzyme in solution) are now being overcome by a new microvolume reaction chamber in a flow condition and with the enzyme immobilized by the Langmuir–Blodgett technique on a glass coverslip activated by a silanizing agent (manuscript in preparation).

## Conclusions

In this work the deposition and the characterization of the GST enzyme are illustrated. The protein structure is slightly modified after LB deposition, but these changes do not significantly affect the enzymatic activity of the enzyme in the film. As written above, the protein denaturation in the LB film at the air–water interface is due mainly to the surface tension of water. The difference in the CD signal between film and solution could be due either to the denaturation of some amount of proteins at the air–water interface or to the formation at the air–water interface of a layer of denatured proteins. This denatured layer would decrease the water surface tension, protecting from denaturation the lowest protein layer. From CD alone it is impossible to discriminate between these two possibilities. In order to exclude the second possibility we have determined the amount of enzyme per each added layer by nanogravimetry (Figure 3), pointing to a constant increase of about  $2.6 \text{ ng mm}^{-2}$  of GST which corresponds to about  $2800 \text{ \AA}^2$ /per GST molecule, which is slightly less than expected by crystallographic data.

It is then more likely that the apparent change in protein conformation, as determined by CD, could be the result of the mutual interaction of the GST molecules when highly packed in the LB film.

In any case, our CD analysis of the GST LB film is compatible with the fact that the film formation and deposition does not change the protein overall structure dramatically, suggesting the preservation of enzyme activity, as indeed shown in Figure 5 and Table 2. The CD spectra were also taken at different surface pressures, namely at 10 and  $35 \text{ mN m}^{-1}$  (Figure 2), in order to determine, along with the apparent film stability and protein packing, the optimal surface pressure for LB deposition (which turned out to be  $20 \text{ mN m}^{-1}$ ).

The nanogravimetric assay also shows that the deposition is reliable, and the activity assays show that the functionality of the enzyme is still maintained up to 423 K. The thermal stability of the LB film with respect to the spread film was significantly increased by optimizing the film formation and deposition procedure.

The preliminary work on the PAB system indicates that the system is able to work reliably with GST in solution in the range between 0.025 and 2 mM.

Therefore, this work can be considered useful and a starting point for any future application of a GST LB film to the PAB system for the detection of electrophilic pesticides (e.g. atrazine).

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