

Ellipsometry as a tool to study protein films at liquid-solid interfaces

Citation for published version (APA):

Cuypers, P. A., Hermens, W. T., & Hemker, H. C. (1978). Ellipsometry as a tool to study protein films at liquid-solid interfaces. *Analytical Biochemistry*, 84(1), 56-67. [https://doi.org/10.1016/0003-2697\(78\)90483-9](https://doi.org/10.1016/0003-2697(78)90483-9)

Document status and date:

Published: 01/01/1978

DOI:

[10.1016/0003-2697\(78\)90483-9](https://doi.org/10.1016/0003-2697(78)90483-9)

Document Version:

Publisher's PDF, also known as Version of record

Please check the document version of this publication:

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Ellipsometry as a Tool to Study Protein Films at Liquid-Solid Interfaces

P. A. CUYPERS, W. TH. HERMENS, AND H. C. HEMKER

*Department of Biophysics, Biomedical Centre, State University Limburg,
Maastricht, The Netherlands*

Received October 5, 1976; accepted August 12, 1977

A description is given of an ellipsometer, automated by means of computer-steered stepping motors. Conversion of analyzer and polarizer readings into thickness and refractive index is done by a graphic procedure on $(\Delta, \psi) \rightarrow (n, d)$ conversion plots. The method is tested by means of Langmuir-Blodgett monolayers of barium stearate. Adsorption of fibrinogen and albumin onto hydrophilic and hydrophobic chromium surfaces, the interaction of fibrinogen with anti-fibrinogen, and the interaction of albumin with anti-albumin are shown as illustrations of the technique.

The ellipsometer is an optical instrument that measures the changes in polarization state of a light beam upon reflection. Any bare reflecting surface will bring about such changes, the magnitude of which are determined by the optical properties of the surface. When a thin dielectric layer is deposited on the reflecting surface the state of polarization is again changed. These changes are directly related to thickness and refractive index of the dielectric layer. The dielectric layer may be a monolayer or multilayer of proteins and lipids. This makes the ellipsometer developed specifically for biochemical work. For a general description and the theory of ellipsometry the reader is referred to Bashara *et al.* (1).

The instrument consists of a light source, polarizer, quarter-wave retarder, reflecting surface, analyzer, and light detector. The parameters Δ and ψ are determined from azimuthal angle measurements of polarizer, analyzer, and compensator at minimal transmission. They are related to the optical constants of the surface, i.e., the thickness and refractive index of the dielectric layer covering it, by the Fresnel reflection coefficients (1).

Until very recently the commercially available ellipsometers were manual instruments; with these instruments normally each measurement takes about 3-4 min. This is too long to follow adequately most biological adsorption processes. Continuous recording of the light intensity at the detector (2) measures only one parameter that is interpretable

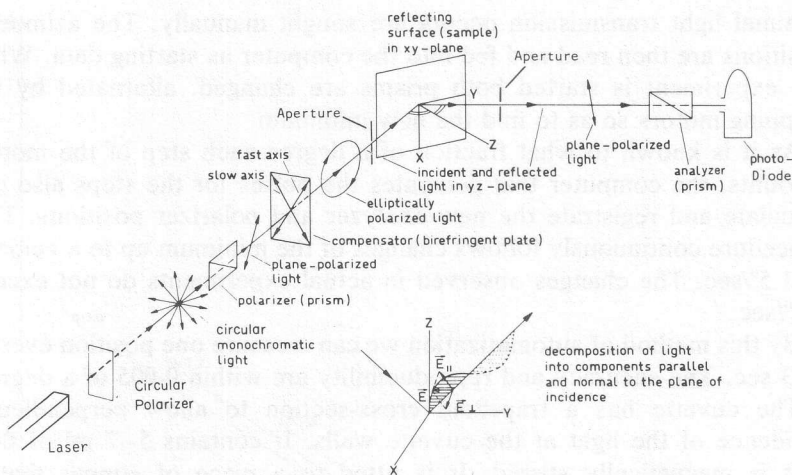


FIG. 1. Principle of the ellipsometer technique.

under very restricted circumstances only in terms of thickness or refractive index of the adsorbed layer.

An automated ellipsometer for biochemical work should have a sensitivity of about 0.005° as this under usual conditions corresponds to a layer thickness of 5 Å. With this sensitivity the resolution of the instrument permits monomolecular layers of biomolecules to be observed. Each measurement can take up to 4 sec as adsorption processes and interactions of biomolecules most often occur in a time scale of minutes.

Commercial automated ellipsometers are either very fast and insensitive or very sensitive and slow (3). The "biochemical" specifications cannot be achieved by trivial changes in these instruments. Therefore we tried a novel approach.

INSTRUMENT

As a basic instrument we used a Rudolph ellipsometer Type 43304-200 E. A He-Ne laser replaced the original light source. Because it was more stable and gave a higher signal at the detector it markedly improved the signal noise ratio. The laser gives linearly polarized light of a fixed azimuthal angle. This would make impossible the function of the polarizer of the instrument. Therefore the laser beam was circularly polarized first. A sketch of the instrument is shown in Fig. 1.

The quarter-wave plate was changed to a compensator that was quarter wave for the He-Ne laser; it was purchased from Lasermetrics. Both polarizer and analyzer were equipped with stepping motors (Fig. 2) that can be computer steered. One step is equivalent to a 0.005° change.

Before zero time the positions of the analyzer and polarizer at which

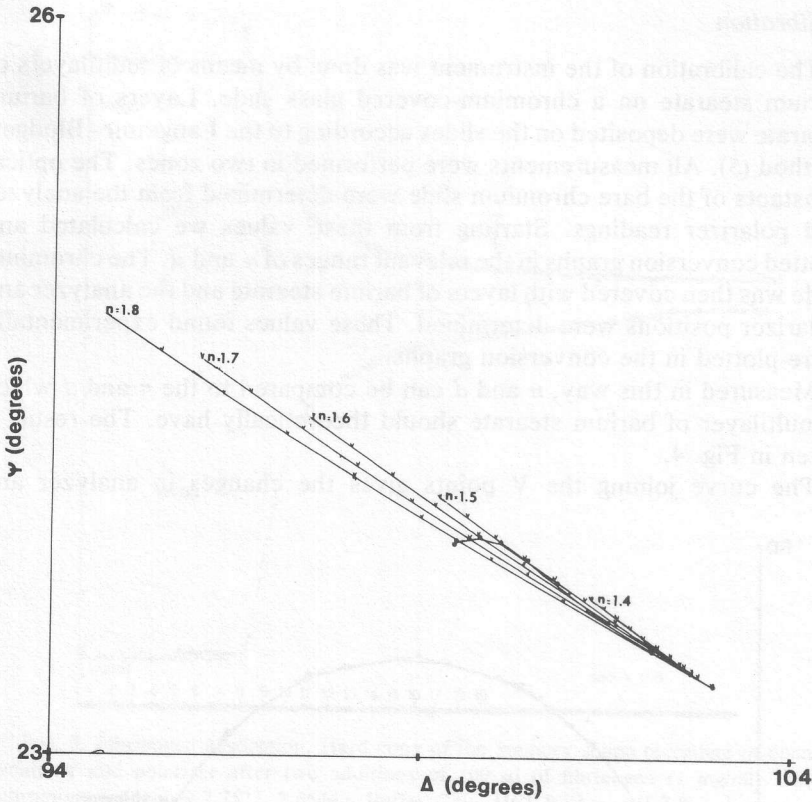


FIG. 3. Conversion graph of fibrinogen adsorption onto hydrophobic chromic surfaces (see text).

We therefore made computer programs that can convert analyzer and polarizer data into thickness and refractive index. With this program any number of dielectric layers can be taken into account and anyone of these layers can be treated as the variable one, once the optical constants of the others are known.

The program simulates the theoretical dependence of analyzer and polarizer of any set of layers on any surface. Because the Drude equations are difficult to solve in a closed form the program calculates analyzer and polarizer settings to a large series of thicknesses and refractive indexes under the prevailing experimental conditions. In this way a map is created from which the thickness and refractive index can be read from any polarizer and analyzer setting to be expected. This map can be represented in the form of a table or a graph. In such conversion graphs (Fig. 3) the experimental data (Δ and ψ values) can be plotted, and thickness and refractive index of the layer can be read directly.

Calibration

The calibration of the instrument was done by means of multilayers of barium stearate on a chromium-covered glass slide. Layers of barium stearate were deposited on the slides according to the Langmuir-Blodgett method (5). All measurements were performed in two zones. The optical constants of the bare chromium slide were determined from the analyzer and polarizer readings. Starting from these values we calculated and plotted conversion graphs in the relevant ranges of n and d . The chromium slide was then covered with layers of barium stearate and the analyzer and polarizer positions were determined. These values found experimentally were plotted in the conversion graphs.

Measured in this way, n and d can be compared to the n and d which a multilayer of barium stearate should theoretically have. The result is given in Fig. 4.

The curve joining the V points gives the changes in analyzer and

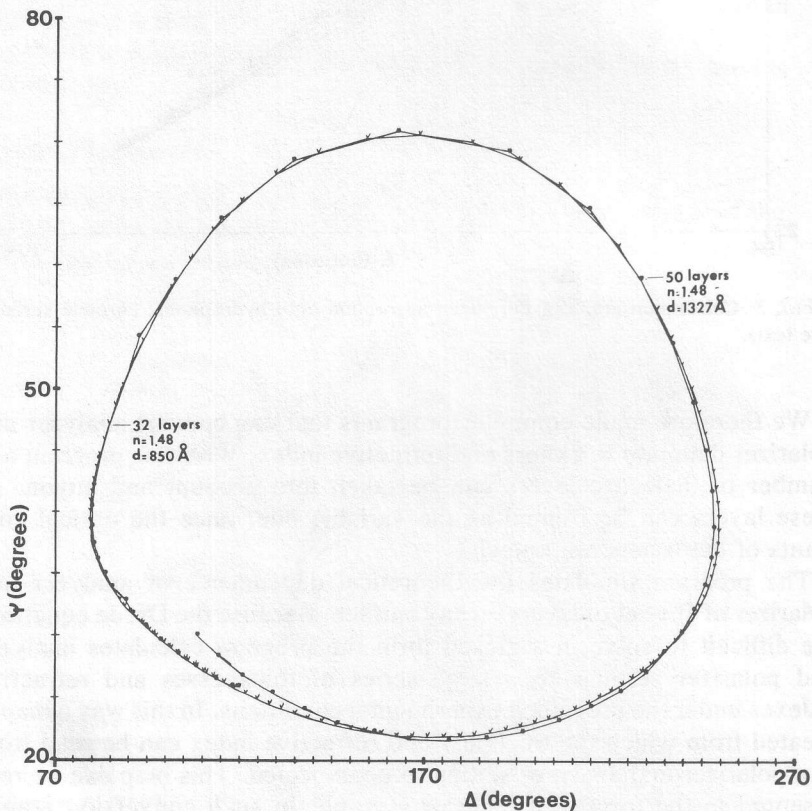


FIG. 4. Barium stearate layers on the effective surface of chromium and the first three layers. Refractive index $n_{\text{eff}} = 2.1092 - 2.3685 i$.

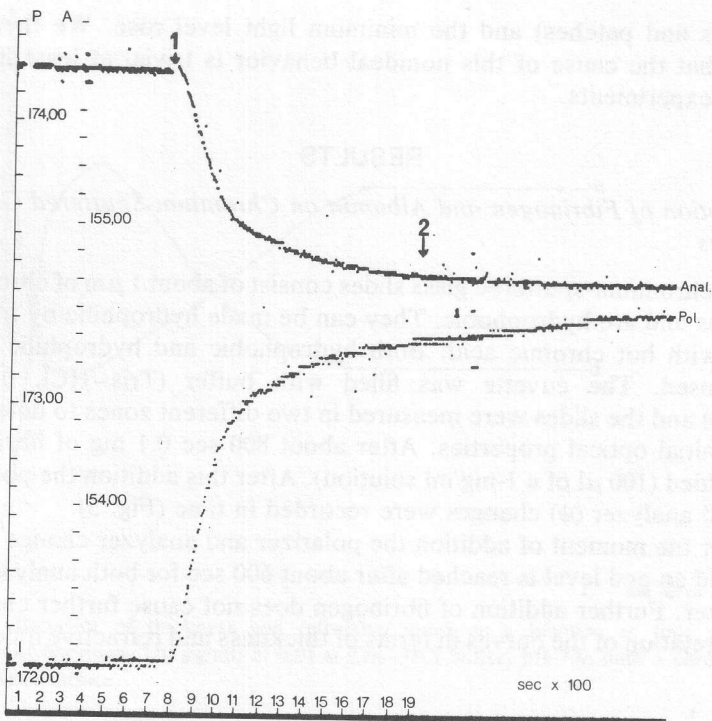


FIG. 5. Fibrinogen adsorption. Hard copy of the memory scope recording of change in analyzer and polarizer after two additions of $100 \mu\text{l}$ of fibrinogen (1 mg/ml). Surface: chromium oxide $n = 2.7527-2.6544 i$. Buffer: Tris-HCl, 0.01 M , $\text{pH } 7.0$.

polarizer when a layer of refractive index $n = 1.48$ grows on the effective chromium surface ($n = 2.1092$, $k = 2.3685$), until a thickness of 300 \AA in steps of 25 \AA each is reached. The black dots show the experimental points of 101 monolayers of barium stearate.

The experimental curve fits the theoretical one reasonably well. The first part of the curve fits exactly when we start the simulation on the effective refractive index of the chromium layer including the first three monolayers of barium stearate. This indicates that the first three layers probably have optical properties different from those of bulk Ba-stearate as is to be expected from theory (6,7).

The experimental curve fits the theoretical one until a thickness of about 50 monolayers, about 1400 \AA , is reached.

After this 1400 \AA the curve starts to deviate from the theoretical values. These deviations might be explained by the slight anisotropy of the layer (6,7). In our experiments, however, these thick layers were several hours old on account of the long procedure necessary to deposit the monolayers. Perceptible disturbances of the layers could be observed

(blisters and patches) and the minimum light level rose. We therefore think that the cause of this nonideal behavior is trivial at least in part of our experiments.

RESULTS

Adsorption of Fibrinogen and Albumin on Chromium-Sputtered Glass Slides

The chromium-sputtered glass slides consist of about $1\ \mu\text{m}$ of chromium on glass and are hydrophobic. They can be made hydrophilic by treating them with hot chromic acid. Both hydrophobic and hydrophilic slides were used. The cuvette was filled with buffer (Tris-HCl, 0.01 M, pH 7.0) and the slides were measured in two different zones to determine their initial optical properties. After about 800 sec 0.1 mg of fibrinogen was added ($100\ \mu\text{l}$ of a 1-mg/ml solution). After this addition the polarizer (Δ) and analyzer (ψ) changes were recorded in time (Fig. 5).

After the moment of addition the polarizer and analyzer change rather fast and an end level is reached after about 600 sec for both analyzer and polarizer. Further addition of fibrinogen does not cause further changes. Interpretation of the curves in terms of thickness and refractive index as a

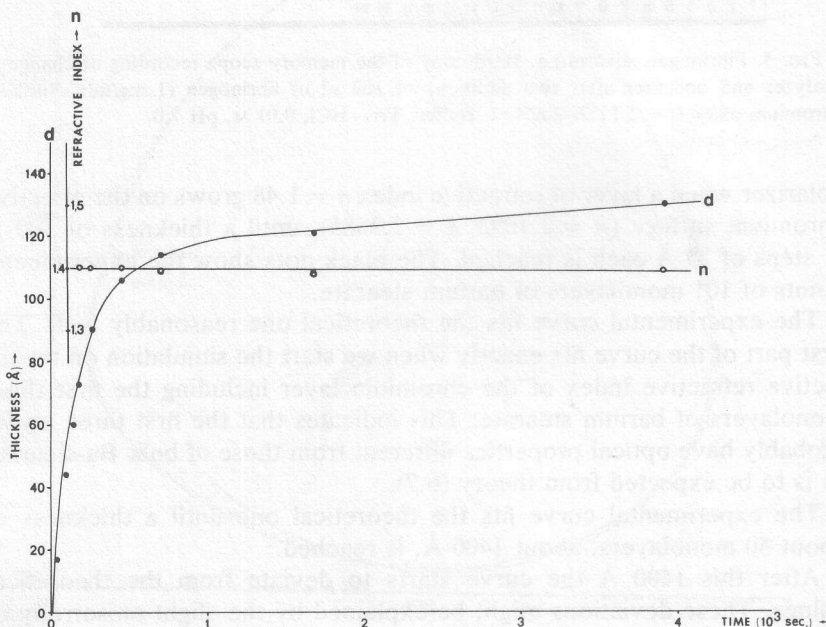


FIG. 6. Behavior of thickness and refractive index as a function of time during the adsorption of fibrinogen ($10\ \mu\text{g}/\text{ml}$) in 0.01 M Tris-HCl, pH 7.0, onto a chromium oxide (hydrophilic) surface.

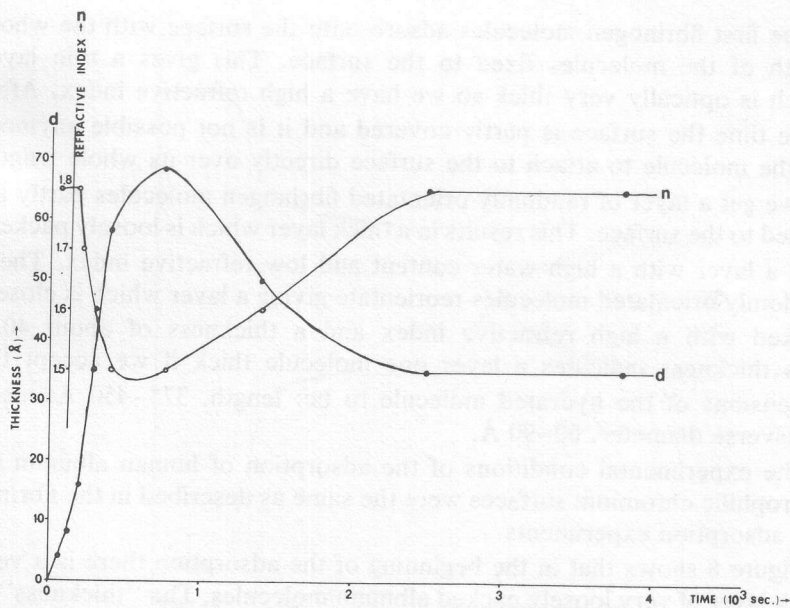


FIG. 7. Behavior of thickness and refractive index as a function of time during adsorption of fibrinogen ($10 \mu\text{g/ml}$) in 0.01 M Tris-HCl buffer, pH 7.0, onto a chromium (hydrophobic) surface.

function of time is given in Fig. 6. This figure shows that with the hydrophilic chromium slides the refractive index does not change much during the adsorption.

The thickness follows the saturation behavior of a monolayer adsorption [type I of Winterbottom (8)]. After about 4000 sec the thickness stabilizes around 130 \AA ; the refractive index is then 1.38. Figure 7 shows the adsorption of fibrinogen on a hydrophobic chromium slide. The experimental conditions were the same as in the foregoing adsorption. During the first 150 sec the thickness grows at a constant refractive index $n = 1.8$. Then the refractive index decreases and the thickness still increases. The thickness increases until about 68 \AA , while the refractive index decreases to about $n = 1.5$. From this point on the layer becomes thinner and optically denser. The layer stabilizes at $d = 35 \text{ \AA}$ and $n = 1.8$.

Conclusions from the Fibrinogen Adsorption Experiments

The difference in character of adsorption on hydrophobic and hydrophilic surfaces depends upon the interaction of the protein and the surface. The interaction with hydrophobic surfaces is much stronger than with hydrophilic ones. This is to be expected as a hydrophilic surface to a certain extent resembles the natural surroundings of a protein in solution. Our explanation of the adsorption behavior is as follows.

The first fibrinogen molecules adsorb onto the surface with the whole length of the molecules fixed to the surface. This gives a thin layer which is optically very thick so we have a high refractive index. After some time the surface is partly covered and it is not possible anymore for the molecule to attach to the surface directly over its whole length. So we get a layer of randomly orientated fibrinogen molecules partly attached to the surface. This results in a thick layer which is loosely packed, i.e., a layer with a high water content and low refractive index. These randomly orientated molecules reorientate giving a layer which is closely packed with a high refractive index and a thickness of about 40 Å. This thickness indicates a layer one molecule thick if we accept the dimensions of the hydrated molecule to be: length, 375–450 Å; mean transverse diameter, 60–90 Å.

The experimental conditions of the adsorption of human albumin on hydrophilic chromium surfaces were the same as described in the fibrinogen adsorption experiments.

Figure 8 shows that in the beginning of the adsorption there is a very thick layer of very loosely packed albumin molecules. The "thickness" is

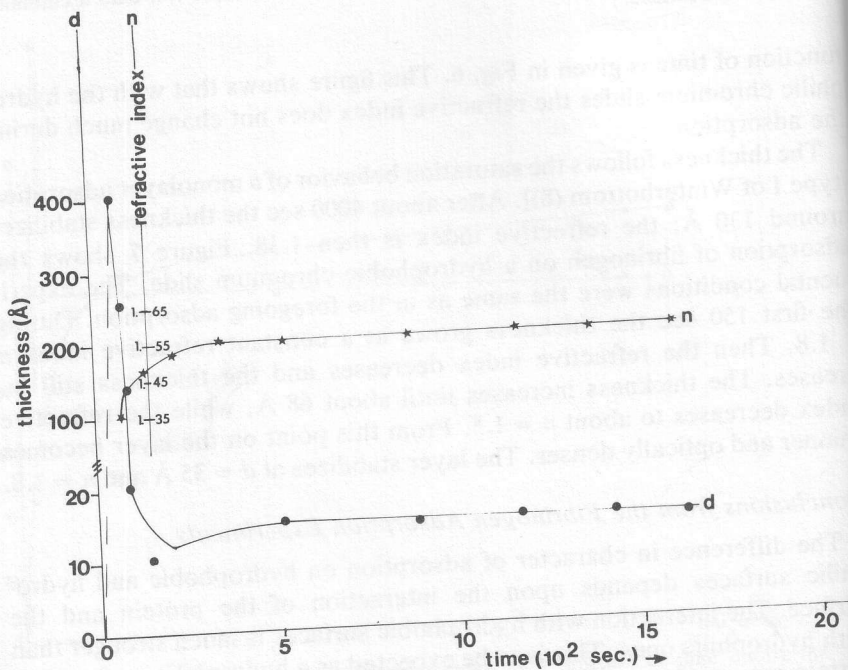


FIG. 8. Adsorption of albumin onto a hydrophilic chromium surface. Concentration albumin: 10 μ g/ml. Buffer: 0.01 M Tris-HCl, pH 7.0.

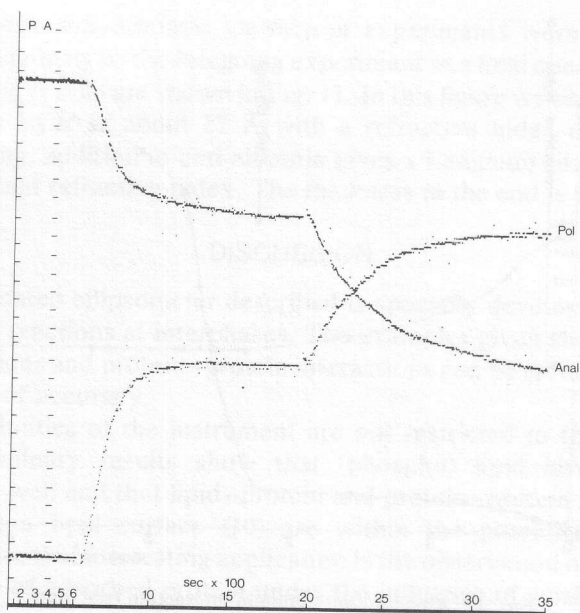


FIG. 9. Fibrinogen-anti-fibrinogen interaction (see text).

about 400 \AA with a refractive index which is only slightly higher than the refractive index of water.

Then rearrangement takes place and a layer which has an average thickness of about 15 \AA is formed. The refractive index is about $n = 1.55$.

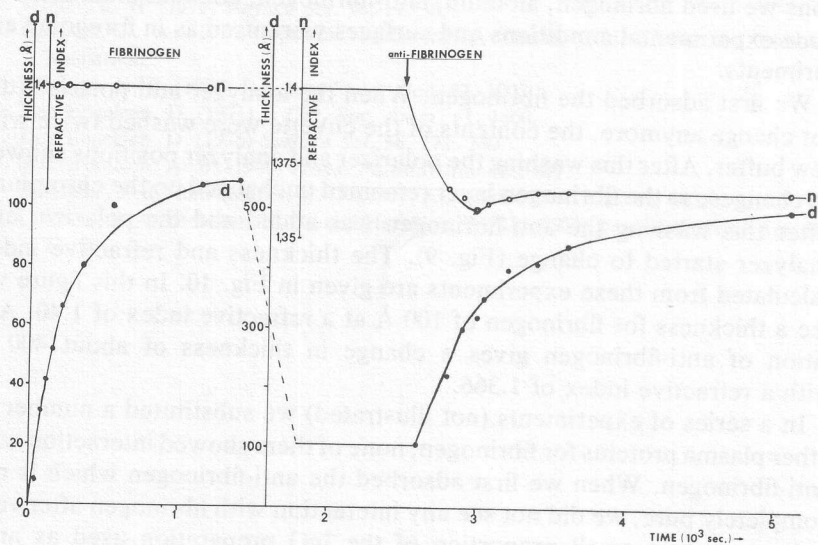


FIG. 10. Fibrinogen-anti-fibrinogen interaction (see text).

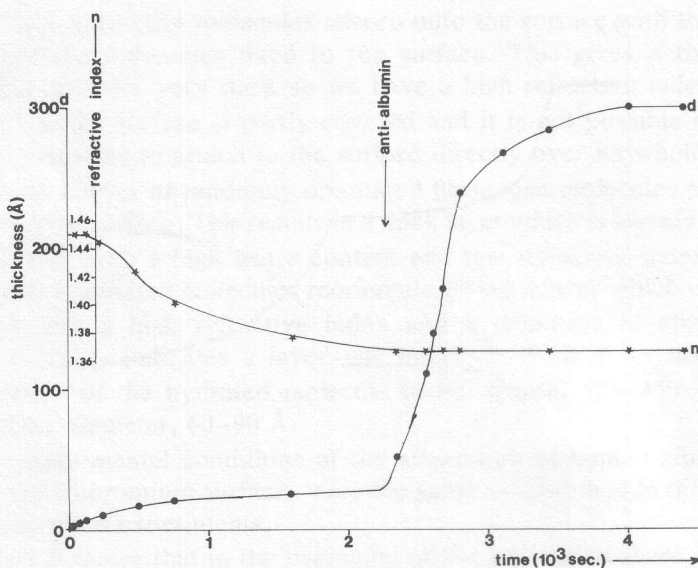


FIG. 11. Albumin-anti-albumin interaction (see text).

This correlates well with the reported dimension of albumin, $21.5 \times 50 \times 106 \text{ \AA}$ (9). These data show how this ellipsometer can be used to measure the adsorption kinetics of proteins and the optical properties of the resulting layers. The instrument can also serve in the investigation of protein-protein interactions at interfaces. For the study of these interactions we used fibrinogen, albumin, anti-fibrinogen, and anti-albumin. The same experimental conditions and surfaces were used as in foregoing experiments.

We first adsorbed the fibrinogen. When the analyzer and polarizer did not change anymore, the contents of the cuvette were washed twice with new buffer. After this washing the polarizer and analyzer positions showed no changes, so the fibrinogen layer remained unchanged on the chromium. After this washing the anti-fibrinogen was added and the polarizer and analyzer started to change (Fig. 9). The thickness and refractive index calculated from these experiments are given in Fig. 10. In this figure we see a thickness for fibrinogen of 100 \AA at a refractive index of 1.40. Addition of anti-fibrinogen gives a change in thickness of about 400 \AA with a refractive index of 1.366.

In a series of experiments (not illustrated) we substituted a number of other plasma proteins for fibrinogen; none of them showed interaction with anti-fibrinogen. When we first adsorbed the anti-fibrinogen which is not completely pure, we did not see any interaction with fibrinogen afterward because only a small proportion of the IgG preparation used as anti-fibrinogen actually is anti-fibrinogen.

The albumin-anti-albumin interaction experiments were carried out exactly analogously to the foregoing experiment at a final concentration of 10 $\mu\text{g}/\text{ml}$. The results are shown in Fig. 11. In this figure we see a thickness for albumin layer of about 22 \AA with a refractive index of $n = 1.447$. After washing, addition of anti-albumin gives a Langmuir-like adsorption with a constant refractive index. The thickness in the end is about 300 \AA .

DISCUSSION

The automated ellipsometer described is specially developed to follow biochemical reactions at interphases. The examples given show that protein adsorptions and protein-protein interactions can be monitored with a high degree of accuracy.

The possibilities of the instrument are not restricted to the examples given. Preliminary results show that (phospho) lipid layers can be measured as well and that lipid-protein and protein-protein interactions occurring at a lipid surface (10) are within the possibilities of this technique. Another interesting application is the observation of conformation changes of adsorbed protein under the influence of small molecular ligands.

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