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Interaction of Blood Proteins With Solid Surfaces

L. E. Smith, W. H. Grant and R. E. Dehl

Polymers Division
Institute for Materials Research
National Bureau of Standards
Washington, D.C. 20234

July 31, 1977

Annual Report for Period
August 16, 1976 to July 30, 1977

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Prepared for

Biomaterials Program
Division of Heart and Vascular Diseases
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Bethesda, Maryland 20014

Interagency Reimbursable Agreement

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Certain commercial equipment is identified in this report in order to adequately specify the experimental procedure. In no case does such identification imply recommendation or endorsement by the National Bureau of Standards nor does it imply that the equipment identified is the best available.

TABLE OF CONTENTS

FIGURES

TABLES

ABSTRACT

The purpose of this investigation is to help develop methods for characterizing the surface properties of cardiovascular implant materials. It is expected that these properties, which are related to the success or failure of implants, will ultimately form the basis for *in vitro* testing of prospective implant materials. The investigation is focused upon three aspects of blood protein adsorption on surfaces, namely (1) the total amount of protein adsorbed, (2) the spatial distribution of adsorbed protein on the surface, and (3) the conformation of adsorbed protein molecules. A number of materials produced by contractors to the NHLBI Biomaterials Program have been examined by ellipsometry and by the adsorption of radio-labeled proteins. Autoradiography has been used to study the uniformity of protein adsorption on several surfaces. Evidence for the migration of proteins into biomaterials has been discovered. Adsorption studies of γ-globulin on a specially prepared oxidized silicon surface have contributed to our basic understanding of the adsorption of blood proteins on surfaces.

SUMMARY

Work on this project is based on the premise that quantitative measurements on adsorbed proteins can serve as methods of surface characterization to aid in the selection and design of materials for implant use and as the basis for the development of *in vitro* test methods.

Protein adsorption is the first in a complex series of events that occurs when blood contacts the surface of an implanted device. The detailed nature of this adsorption, i.e., the proteins involved, their absolute and relative amounts, the conformational changes of the proteins, the number and types of binding sites, and the spatial distribution of the protein across the surface, potentially affect the later stages of the interaction of the formed elements with the implant.

Accomplishments in this past year are described below.

1. CHARACTERIZATION OF NHLBI CONTRACTOR'S SAMPLES

Adsorbed Protein Conformation Measurements

Samples from six NHLBI contractors have been examined. Fibrinogen adsorption measurements have been made by ellipsometry on 3 of these samples, supplying data relating to the conformation of the adsorbed protein as well as the amount adsorbed. Radiotracer measurements of labeled human serum albumin have been used to clarify the ellipsometric data.

Measurement of the spatial distribution of adsorbed protein

Non-uniform protein adsorption can be diagnostic for sites of high thrombogenetic potential that may be difficult to visualize in any other

manner. Certainly it can be used as a quality control method for evaluation of surface properties. Inhomogeneous surfaces may be responsible for failure in in vivo tests when the average properties of the surface seem acceptable in in vitro criteria.

During the past year we have carried out a preliminary survey of the adsorbed protein distribution on a number of NHLBI contractor's samples using autoradiography. The method has been shown to be useful and is capable of revealing subtle surface features.

Preliminary data on albumin adsorbance on polyurethanes has led us to propose that such materials are significantly permeable to blood proteins. Verification of this fact in regard to polyurethanes and other contractors samples is currently in progress.

2. INVESTIGATION OF MODEL SURFACES

Investigation of radiotracer methods of protein adsorption measurements

The effects of protein purity have been investigated and suitable separation and purification methods for labeled human serum albumin have been developed. Different labeling isotopes, ^{131}I or ^{125}I have been used and shown to yield the same results. The higher energy ^{131}I decay has been considered a likely cause of protein fragmentation by radiolysis. Such a mechanism has been shown to be of no consequence for short term adsorption measurements.

Investigation of the surface properties responsible for non-uniform adsorption

Strikingly non-uniform protein adsorption has been observed on a platinum surface. Several potential causes for this distribution can be proposed,

including microcrack formation and enhanced adsorption due to stress history. These latter two mechanisms have implications for similar effects in biomaterials. The technique of autoradiography is therefore of significant potential value to the Biomaterials program and a major part of next year's effort will be devoted to its application to contractors samples and control studies of other reference surfaces.

Progress during the past year has been quite close to the milestones outlined in the negotiated program plans. The only notable exception is the secondary ion mass spectrometry (SIMS) investigation which is considerably behind schedule due to severe equipment problems outside our control. These problems have not been satisfactorily resolved and the SIMS work for next year will be largely curtailed. Major emphasis will be placed on autoradiographic measurement of surface homogeneity and measurement of protein penetration into the biomaterial. All other aspects of the negotiated program plan will also be pursued on schedule.

biomaterials with blood. A suitable set of experimental criteria established by these studies may result in rapid *in vitro* screening tests for candidate biomaterials.

INTRODUCTION

The adsorption of plasma proteins is the first in a complex series of events that occurs when a synthetic material is placed in the cardiovascular system. It is known that this protein adsorption can alter the course of subsequent reactions at the implant surface but the mechanism by which this occurs is not well understood. If the detailed behavior of proteins adsorbed at the solid surface were known and capable of control, the fate of artificial materials placed in body could be predicted with greater certainty and more successful materials perhaps could be designed. This investigation is directed toward the identification of factors capable of affecting protein adsorption and the development of a description of the changes that occur in the protein upon interaction with a surface.

We have continued our examination of potentially useful synthetic implant materials provided by contractors to the NHLBI Biomaterials Program. In addition to the ellipsometric studies of fibrinogen adsorption on materials whose optical properties were suitable for such investigation, we have measured the adsorption of radiolabeled human serum albumin on several of these materials. During the course of this work, it has become apparent that some synthetic biomaterials may be permeable to proteins, which could significantly affect our interpretation of the ultimate success or failure of these materials in contact with the blood. Autoradiography studies have also been initiated, both to serve as a qualitative confirmation of the radiotracer counting method, and to test for non-uniform spatial adsorption of proteins on surfaces. These studies are expected to lead to a better understanding of surface properties governing the compatibility of synthetic

EXPERIMENTAL

Protein Purity

Fibrinogen

The human fibrinogen used in this work was purified by us according to the Batt-Laki (1,2) method, as reported in our previous Annual Reports (3-6), yielding a product which is greater than 96.7% clottable. Solutions of the purified protein were quick-frozen in liquid nitrogen and stored at low temperature until needed. Future experiments will utilize the commercially purified fibrinogen supplied to all contractors to the NHLBI Biomaterials Program

γ-Globulin

Bovine γ-globulin (Cohn Fraction II) had an electrophoretic purity of 95% as stated by the supplier. The purity of this material was tested by fractionationation through a Sephadex G-200* gel permeation chromatography column, and the results are reported in the following section of this Report.

The radiotracer adsorption experiments utilized both tagged and untagged HSA. The protein was tagged with a labeling atom of ^{125}I or ^{131}I by commercial sources. Additional purification was conducted in our laboratory by passing both labeled and un-labeled materials through a Sephadex G-200 gel permeation chromatographic column which separated out the higher molecular weight aggregates, dimer, monomer and low molecular weight polypeptides. The chromatographic separation of these materials is reported in a separate

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section of this report.

Ellipsometry

Ellipsometric studies of fibrinogen adsorption on materials supplied by contractors to the NHLBI Biomaterials Program was conducted in pH 7.4 buffer solution at 37°C. All γ-globulin experiments, using both manual and automatic ellipsometers, were carried out at ambient room temperature.

Both manual and automatic ellipsometers employ a He-Ne laser light source (λ = 632.8 nm) with an incident angle of 70°. For the manual ellipsometer, determination of the polarizer (P) and analyzer (A) settings necessary to produce extinction of the reflected light from the surface was performed by the method of "swings", in which a photomultiplier with a voltmeter output is used to determine the null point by setting P or A so as to produce equal light intensity on both sides of the null point. Since the plot of reflected light intensity vs. P or A setting is parabolic about the null point, a much more accurate null can be determined by averaging the readings taken at the steep portion of the parabolic curve on both sides of the null, than by simply measuring the null directly. This method allows us routine measurements of polarizer and analyzer settings with an angular precision of $\pm$ 0.01°. The automatic ellipsometer employs a rotating analyzer to measure the ellipticity and azimuth of the reflected light directly at a constant polarizer setting, rather than by adjusting P and A for reflected null. Both variables are presently being recorded on a dual-channel strip chart recorder. All solution experiments were performed in specially designed cells, with windows tilted at the 70° incident angle in order to provide normal incidence of light upon the windows and thus eliminate refraction of light by the cell. Further details of the ellipsometry instrumentation and experimental proceedings may be found in previous Annual Reports (3-6).

Analysis of the ellipsometry data to determine protein film thickness, refractive index, and the amount adsorbed on the substrate was performed with a computer program developed at NBS by Dr. F. L. McCrackin (9).

Radiotracer Counting

A highly efficient γ-counting system was assembled with commercially available components and used to count the human serum albumin labeled with ^{125}I and ^{131}I. The detector was a high-resolution 76.2 x 76.2 mm NaI crystal scintillation detector. Photomultiplier power was supplied by a high voltage DC regulated power supply. Photocurrent from the photomultiplier was amplified and fed to a single channel analyzer, with adjustable window and baseline controls which were optimized for either ^{125}I or ^{131}I to yield the maximum signal count and minimum background count.

With this simple and relatively inexpensive system, we have been able to achieve a counting efficiency comparable to that of well known commercial γ-counters. In order to test the efficiency of our system, we counted a calibrated sample of $K^{125}I$ in carrier solution (SRM 4407L-B) supplied by the Office of Standard Reference Materials at NBS. In addition, through the courtesy of Dr. Henriksen at the National Cancer Institute (NIH), we counted samples of human serum albumin labeled with ^{125}I and with ^{131}I, on a commercial counting system. The efficiency of our system proved to be somewhat greater than that of the commercial system.

The experimental techniques which we have developed for performing quantitative adsorption studies of blood proteins, including the design of the adsorption cell and the very important rinsing procedures required to remove radioactive solution carry-out from adsorbates have been described in the Annual Report for 1976 (6), and in previous Annual Reports (3-5).

Specific activity determinations were made as follows. Aliquots of the radioactive solution were pipetted into small test tubes, weighed, and counted with the γ-counter, to yield the sample activity in counts/min/wt. of solution. The density of the solution was assumed to be 1. The solution concentration was determined by measuring the optical density on a UV spectrophotometer. In this way, the specific activity in counts/min/wt. of protein was determined. In order to test for possible self-absorption of radiation by the solution and its container, various experiments were performed in which solutions were counted before and after drying, with and without glass and plastic containers surrounding the sample. The count for a given sample proved independent of any of these factors.

Most adsorption experiments were performed in duplicate, in order to test the reproducibility of our results. We find that our current procedures permit us to replicate a given adsorption experiment within $\pm$ 2%. For many purposes, therefore, it will not be necessary in the future to perform duplicate experiments.

Autoradiography

A number of autoradiographic experiments have been performed during the past year, after our valuable consultation with Dr. Curtis Harris at the National Cancer Institute (NIH). The experiments reported here were performed by pressing x-ray film against both sides of a sample surface which has been covered with radiolabeled protein by adsorption in the usual manner. The surfaces were dried and clamped between glass plates to insure good contact between film and specimen. The purpose of exposing film to both sides, and to allow additional exposure time for one side, if the developed film from the other side indicates insufficient exposure. All autoradiographs described in this report were developed after a two-week

exposure at room temperature. In most cases, normal development times were used, although for some samples, as noted in the following Discussion, the film was deliberately over- or under-developed in order to bring out certain features of the protein distribution on the surface. Optimum exposure times depend upon the radioactivity of the sample, as well as the temperature and humidity of the storage vessel.

Secondary Ion Mass Spectrometry (SIMS)

Secondary Ion Mass Spectrometry (SIMS) is a technique in which a beam of ionized gas as narrow as 1 μm is focussed upon a small surface area, sputtering off a secondary beam of ions from the surface. The secondary beam is collected and analyzed by a mass spectrometer, producing a spectrum characteristic of the material on the surface. By scanning across the surface, it is possible by this technique to determine the homogeneity of the material on the surface, and by continuously sputtering one spot on the surface or scanning a cross section of the material it is possible to produce a depth profile of the atomic and/or molecular composition of the substrate material. The intended uses of this technique in our work have been to study the uniformity of distribution of adsorbed proteins on surfaces and the depth of penetration of the protein into uncoated substrates, hydrogel coatings, heparinized surfaces, etc.

The SIMS apparatus at NBS is located in the Analytical Chemistry Division. Dr. K. F. J. Heinrich, Chief of the Microanalysis Section, and Dr. D. Newbury have agreed to examine polymer substrates with the SIMS technique and have offered to perform preliminary experiments on biomaterial substrates which are of interest to us and to the Project Officer. Unfortunately, as noted in the following discussion, instrumental breakdowns have prevented us from exploiting this technique in a productive way thus far.

NHLBI Contractor Biomaterials

The NHLBI Contractor Biomaterial samples which we have examined this year by ellipsometry and radiotracer methods were all shipped to us in sterile packages. All were shipped dry except for the polyalkylsulfone, one sample of which was shipped under water in a plastic pouch. No surface cleaning procedures were used other than a water rinse prior to ellipsometry or radiotracer studies. The NHLBI contractors have been most cooperative in preparing samples which are suitably thick (1.5-2 mm) for ellipsometric and radiotracer adsorption studies, even though such materials as the membrane oxygenators would normally be fabricated in much thinner sheets than is required for our measurements.

Characterization information and identification of actual samples investigated has been transmitted to the Biomaterials Program, National Heart, Lung and Blood Institute, NIH.

RESULTS AND DISCUSSION

EXAMINATION OF NHLBI CONTRACTOR MATERIALS

All of the candidate materials which we have received from contractors to the NHLBI Biomaterials Program have been examined by ellipsometry, and we have performed fibrinogen adsorption experiments upon all but one of the materials whose optical properties allowed meaningful measurements to be made. The only materials on which adsorption studies have not as yet been performed are the carbon-coated samples from General Atomic Co, as noted below. With this exception, each material listed in our 1976 Annual Report under the heading "Current Status of Other NHLI Contractor Biomaterials" has been studied by us during the past year, and the results are presented below.

As a group, the dielectric substrates on which we have performed ellipsometry studies during the past year present certain difficulties in obtaining precise quantitative information about adsorbed protein films. The surfaces which we receive are not always as flat or smooth as is required to perform quantitative ellipsometry measurements. Further, the refractive index of substrates such as these tends to differ less from that of the overylying protein film than is necessary for precise quantitative measurement of the film properties. Since some of these materials imbibe aqueous solutions there is also the possibility that the refractive index of the substrate may change during an ellipsometry run, which prohibits quantitative interpretation of the observed data. For these reasons, ellipsometry is, at best, a semiquantitative tool for measuring physical properties of protein films on dielectric substrates. However, as we will show, it can be very useful indicator of differences between the adsorptive behavior of various substrates and, in conjunction with radiotracer

measurements can contribute significantly to our overall understanding of the interaction of blood proteins with solid surfaces.

Ethylcellulose Perfluorobutyrate

In our Annual Report for 1976 (6) we reported that ethylcellulose perfluorobutyrate was found to adsorb a much greater amount of fibrinogen after 2-3 h than any other substrate thus far examined. However, the adsorbances noted at 1/2 h and 1 h were about normal (3-5 mg/m^2) as compared with our measured fibrinogen adsorbance on other substrates. Another sample of this material yielded an adsorbance of about 3 mg/m^2 at 1/2 h and 1 h. (For this sample, the experiment was terminated after 1 h). The large increase in adsorbance which we noted for the first 2 samples between 1 h and 2 h is very unusual, for a solution concentration as high as the 3 mg/ml used in these experiments, and we suspect that the substrate refractive index has changed significantly during this interval, leading to the very high calculated adsorbance at 2-3 h.

Two circular samples, cut from the same sheet from which the ellipsometry samples were taken, were equilibrated for 1 h in ^{125}I-labeled human serum albumin (HSA) solution, at a concentration of 7.02 mg/ml. The samples were rinsed, dried and counted with the γ-counter in the usual way. The average of the two yielded an adsorbance of 9.99 mg/m^2. Another sample, equilibrated for 1 h in ^{131}I-labeled HSA solution, at a concentration of 2.95 mg/ml, yielded an adsorbance of 3.99 mg/m^2. Since the adsorbance of HSA on polyethylene discs

from these 2 difference HSA solutions was found to be essentially the same after 1 h equilibration, we do not feel that the differences noted here can be due to specific isotope labeling effects, different for the 2 iodine isotopes.

It is perhaps significant that the ratio of adsorbances for HSA(^{125}I)/(^{131}I) is 2.50, while the corresponding ratio of solution concentrations used in the adsorption experiments is about the same (2.38). These data suggest to us either that the adsorbance on this substrate in the concentration range used here is proportional to the solution concentration, or that there is some permeation of the protein solutions into the substrate, entailing a migration of the protein molecules into the matrix. During the next year, we expect to perform experiments which will test these hypotheses, and we expect that the results will lead to a better understanding of the interaction of protein solutions with biomaterial surfaces.

Polyalkylsulfone

Two samples of the polyalkylsulfone membrane oxygenator material have been examined, and the results are quite different from any that we have observed to date. The changes in reflection coefficients upon exchanging the fibrinogen solution for buffer were so small as to indicate that virtually no fibrinogen was deposited on the surface. While this would seem the most likely explanation for the phenomenon, there is also a possibility that a fibrinogen film was deposited, whose refractive index was essentially identical to that of the underlying substrate. In this case, the incident light

would not "see" the film even though it is there. Since the index of refraction of the substrate (1.47) is considerably higher than that of the protein solution (1.33), the fibrinogen film would have to be quite dense, compared with any other that we have observed thus far. In order to resolve this question, an adsorption experiment was performed from a much more dilute fibrinogen solution (0.12 mg/ml). If a dense film was deposited from the more concentrated solution, a less dense film should be deposited from dilute solution, corresponding to a lower point on the adsorption isotherm, which hopefully could be detected by the ellipsometer. This apparently has proved to be the case. From the more dilute solution, the ellipsometer measured an adsorbance of about 1.7 mg/ml, which increased only slightly from 1/2 h to 2 h adsorption time. While this measurement tends to support the "dense film" hypothesis, it must also be considered in the light of the radiotracer experiments reported below.

Adsorption with Radiolabeled HSA(^{125}I)

Two circular discs cut from the same sheet of PAS used in the ellipsometry experiments were used to study the adsorption of HSA(^{125}I). A solution concentration of 7.02 mg/ml was used, and the adsorption experiments were performed in the usual way, for 1.2 h. The average of 2 determinations of the adsorbance was 19.7 mg/m^2. This result is in sharp constrast with the ellipsometry results for fibrinogen adsorption, reported above, in which one possible interpretation of the data was that no protein was adsorbed on the material. The very high adsorbance of the HSA suggests the possibility that the fibrinogen adsorbance from a solution concentration of 3 mg/ml may also have been quite high.

Glow-Discharge Coatings

Unlike many other coated surfaces which we have examined by ellipsometry, the tetramethyl disiloxane glow-discharge coated polyethylene sample from Research Triangle Institute gave a specular reflection, and three fibrinogen adsorption experiments were performed on it. Two of the three samples gave similar results, while the third gave inconsistent results for the fibrinogen film thickness. The calculated adsorbance for the first two samples were in the range 3.2-4.9 mg/m^2 during the first hour of adsorbance, with little change between 1/2 h and 1 h. One of the samples which was allowed to equilibrate for 2 h apparently adsorbed another 9 mg/m^2 in the interval between 1 h and 2 h. The same phenomenon was noted above for the ethylcellulose perfluorobutyrate and, as we have noted, the apparent adsorbance is probably due to changes in the substrate which render the calculated adsorbance suspect.

Heparin-coated Silastic

Two samples of heparin, bonded to silastic-coated glass with APTES, were examined by ellipsometry. In both cases, light scattering from the surface prohibited measurement of the reflection coefficients with sufficient accuracy to warrant a fibrinogen adsorption measurement. This substrate is a candidate for radiotracer studies, which will be performed in the near future.

Polyetherurethanes

Our ellipsometric study of fibrinogen adsorption on two polyether-urethanes (A) and (B) was discussed in the Annual Report for 1976. We reported that the average fibrinogen film thickness on (A) was 30.7 $\pm$ 4.6 nm, and on (B) was 32.0 $\pm$ 5.7 nm. The average fibrinogen adsorbance on (A) and (B) were found to be, respectively, 4.75 $\pm$ 0.23 mg/m^2 and 5.04 $\pm$ 0.39 mg/m^2. From these data we concluded that there is no significant difference in the adsorbed amount or molecular extension of fibrinogen on the 2 substrates, as measured by ellipsometry. These findings were not unexpected, given the chemical similarity of the two materials, which would be expected to lead to essentially the same protein adsorption affinity. Certain physical differences were noted between the two materials, however. (B) is mechanically less flexible than (A), and, unlike the latter, does not appear to imbibe water after immersion for several hours. These differences may be responsible for the considerable differences in affinity for radiolabeled albumin solution, as reported below.

Two circular discs of (A) and (B) were cut from the same sheets as those from which the ellipsometry samples were taken. One sample of each material was flexed by bending sharply across the disc several times. This flexure prior to protein adsorption was done to test the possibility that the adsorption might be affected by partial orientation of the polymer molecules, resulting from stress orientation. The four samples were equilibrated for 1/2 h in ^{125}I-labeled human serum albumin, at a concentration of 7.02 mg/ml, in our usual way. They were then rinsed, dried overnight in air, and pressed between x-ray film for two weeks, as we have discussed above. Enlarged photographs of the 2-week autoradiographic films of (A) and (B) are shown in Figs. 1 and 2 . The light areas correspond to the radiotracer-activated areas of the film, and both prints were made at the same exposure time. Clearly,

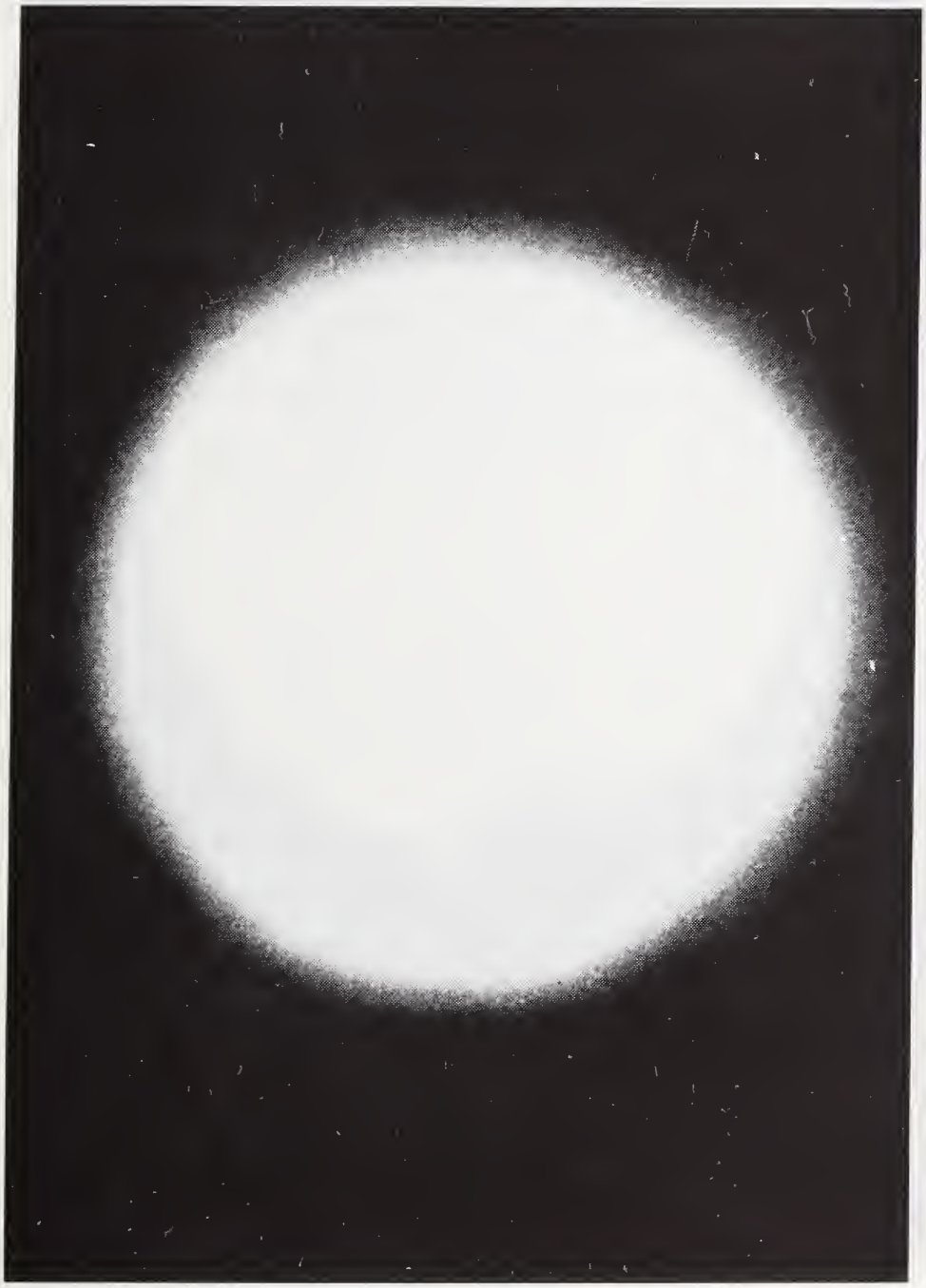

Figure 1. Autoradiograph of polyetherurethane (A) with adsorbed HSA(^{125}I).

Figure 2. Autoradiograph of polyetherurethane (B) with adsorbed HSA(^{125}I).

the (A) substrate has a greater amount of adsorbed HSA than the (B) substrate. There was found to be no discernible difference between the autoradiographs of flexed and unflexed samples of each material, nor was any significant difference noted between the autoradiographs of the 2 sides of a given sample. These two substrates clearly have considerably more adsorbed radiolabeled protein than any other material that we have studied by autoradiography thus far, a result which is confirmed by radiotracer counting as reported below.

Adsorption of both HSA(^{125}I) and HSA(^{131}I) was carried out on circular disc samples of (A) and (B). Adsorption was performed for about 1 h on each sample, followed by our usual rinsing, drying, and counting procedures. The concentration of the HSA(^{125}I) solution was 7.02 mg/ml, while that of the HSA(^{131}I) was 2.95 mg/ml. Average results for two samples of each substrate are as follows. For substrate (A), adsorbance of HSA(^{125}I) was 42.01 mg/m^2, and adsorbance of HSA(^{131}I) was 10.98 mg/m^2. For substrate (B), adsorbance of HSA(^{125}I) was 24.19 mg/m^2, while HSA(^{131}I) adsorbed 5.54 mg/m^2. For both isotopes, therefore, it is clear that adsorbance on the (A) substrate is about twice that of the (B), confirming the autoradiographic results above, which indicated considerably higher adsorbance on (A) than on (B).

It is useful to compare these findings with those reported above for the ethylcellulose perfluorobutyrate. Although the latter substrate adsorbed less HSA(^{125}I) and HSA(^{131}I) than the polyetherurethanes, the adsorbance ratio of the two HSA's was roughly the same for all three materials. We believe that the possibility of a specific isotope labeling effect, different for ^{125}I and ^{131}I, has been eliminated by the adsorption experiments on polyethylene, as reported below. The only significant difference between the adsorption experiments involving the two isotopes, as noted above, was the concentration of the solutions. Since the higher apparent adsorbance in each case corresponded to the higher HSA concentration, and since the equilibrium

adsorbance from both solutions is expected to be about the same, we are led to the conclusion that the "adsorbances" measured here represent, in fact, an entrapment of some protein solution by the materials during the 1/2 h immersion in HSA solution. Solution is trapped by the substrate in a way which prevented its being rinsed off during the brief (2-3s) rinsing time, which has been shown to be adequate for removal of protein solution from nonporous surfaces such as polyethylene or glass.

Carbon

One sample of a graphitized carbon heart-valve disc alloyed with 10% Si was studied by a 2-week autoradiographic exposure, after HSA (^{125}I) adsorption following our usual procedure. Figs. 3 and 4 are the autoradiographs of both sides of the disc. One side (Fig. 3), which was developed for the normal 4.5 minutes, shows an intense line due to a scaratch which was placed on the surface as an aid in orienting the sample prior to HSA adsorption. Enhanced protein adsorption, and possibly incomplete removal of protein solution during the normal rinse time, are responsible for the high radiolabeled protein concentration in the scratch. Except for a small ring of adsorbed protein near the outside rim of the disc, there is very little evidence of adsorbed protein on this side of the disc. The film on the other side (Fig. 4) was developed for only 2-1/2 minutes. Although the light intensity is low, there is clear evidence of protein adsorption over the entire surface, and a number of enhanced adsorption regions. The word "Reject" which was etched into the surface has clearly enhanced protein adsorption, and there is a relatively bright ring near the edge on which adsorption is enhanced.

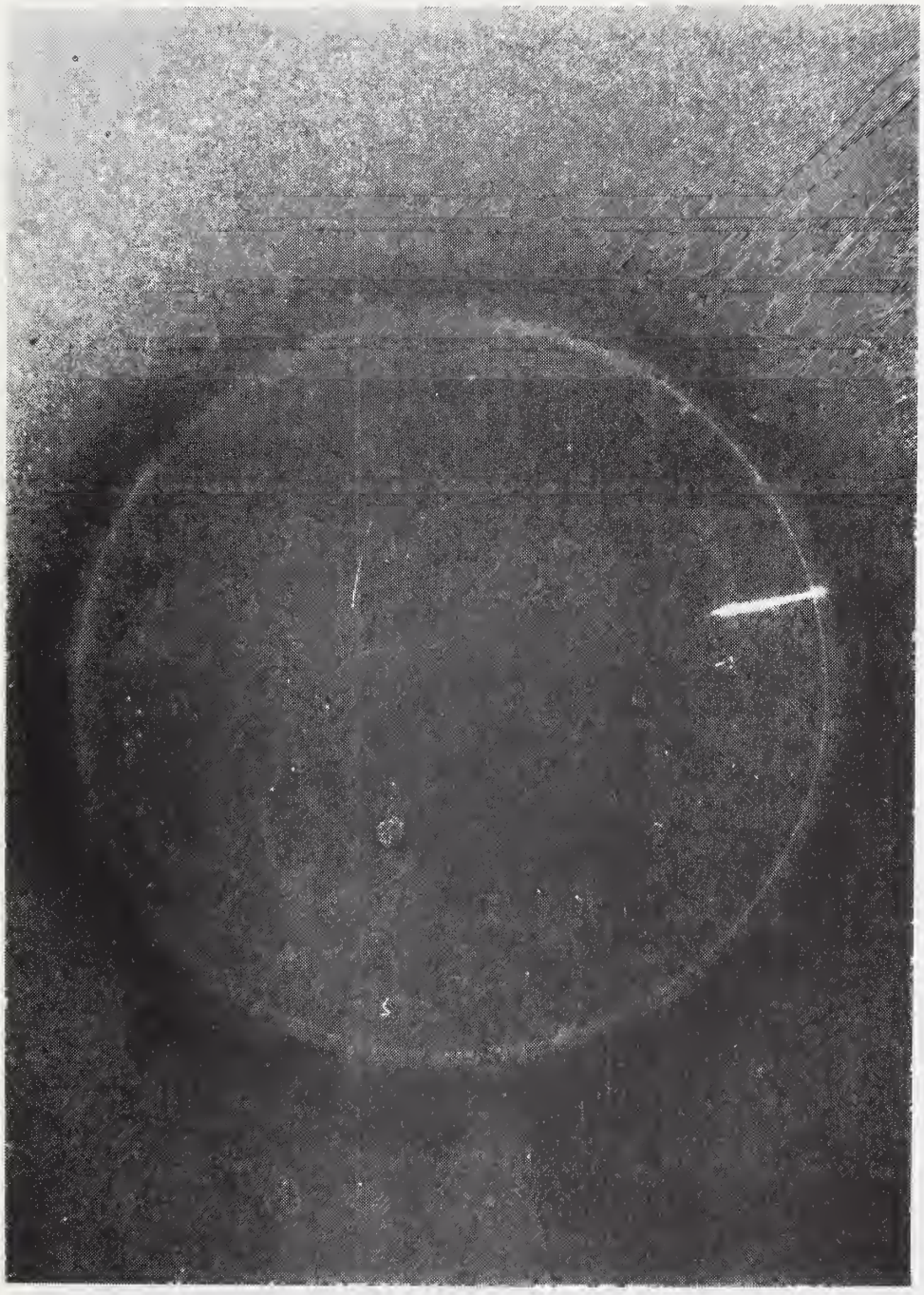

Figure 3. Autoradiograph of carbon disc, with adsorbed HSA(^{125}I).

Figure 4. Autoradiograph of carbon disc, with adsorbed PSA-(^{125}I). Film under developed.

CURRENT STATUS OF OTHER NHLBI CONTRACTOR BIOMATERIALS

All of the NHLBI Contractor Biomaterials which we have received and agreed with the Project Officer to examine have been studied by ellipsometry, except for certain carbon-coated substrates. We are presently awaiting the receipt of fresh samples of these materials.

As noted in the above Discussion, radiotracer and autoradiography are providing valuable information about amounts and distribution of radiolabeled proteins on surfaces. We expect to continue to use both of these techniques to study biomaterial surfaces. Each of the Contractor Biomaterials listed in the report will be further examined, using both of these techniques, with special emphasis on samples such as the heparinized Silastic, which cannot be examined by ellipsometry.

In our last work statement we proposed to use secondary ion mass spectrometry (SIMS) to study protein distribution on surfaces of interest to the Biomaterials Program. This work was to be performed on a spectrometer in the Analytical Chemistry Division at NBS. We have been able to obtain preliminary spectra from a pyrolytic carbon disc alloyed with 10% Si, and of a stainless steel planchette "ion-plated" with carbon. The spectra obtained are shown in Figs. 5 and 6. Clearly, numerous surface impurities are present on these materials, although some additional work would be required to obtain a quantitative analysis of surface impurities by this method. Unfortunately, numerous instrumental breakdowns have prevented further use of the SIMS equipment, and we have not been able to obtain any information as yet on the protein-coated substrates which are of interest to this program.

While the SIMS technique appears less than promising at this time, we believe as a result of recent findings that most of the information that would have been obtained by SIMS about protein distribution on surfaces and depth of penetration into substrates will be furnished by radiotracer techniques, including autoradiography and radiotracer studies of protein migration into biomaterials. As noted below, our preliminary results obtained by these techniques are most encouraging.

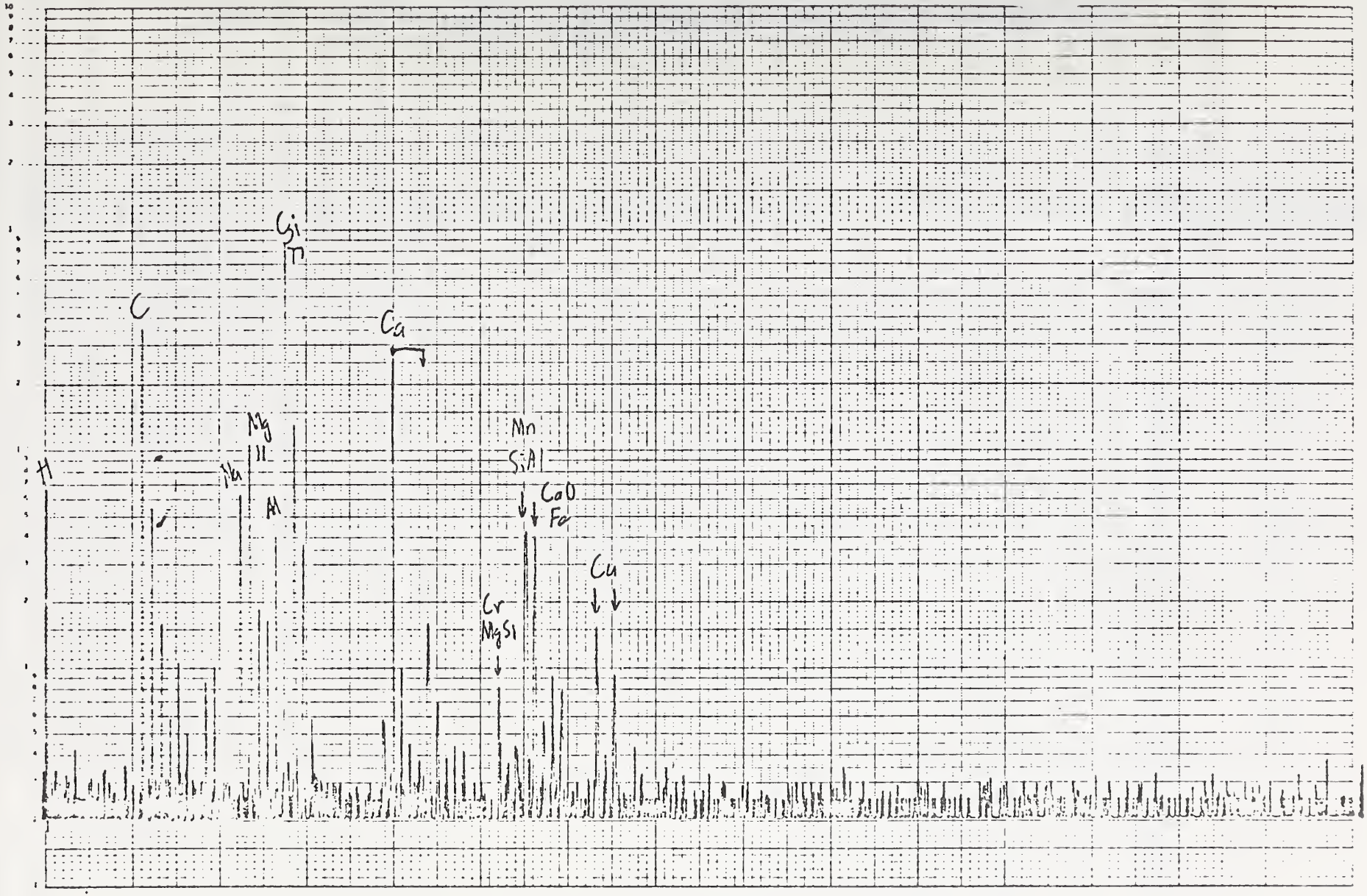

Figure 5. SIMS spectrum of carbon-10% Si disc surface.

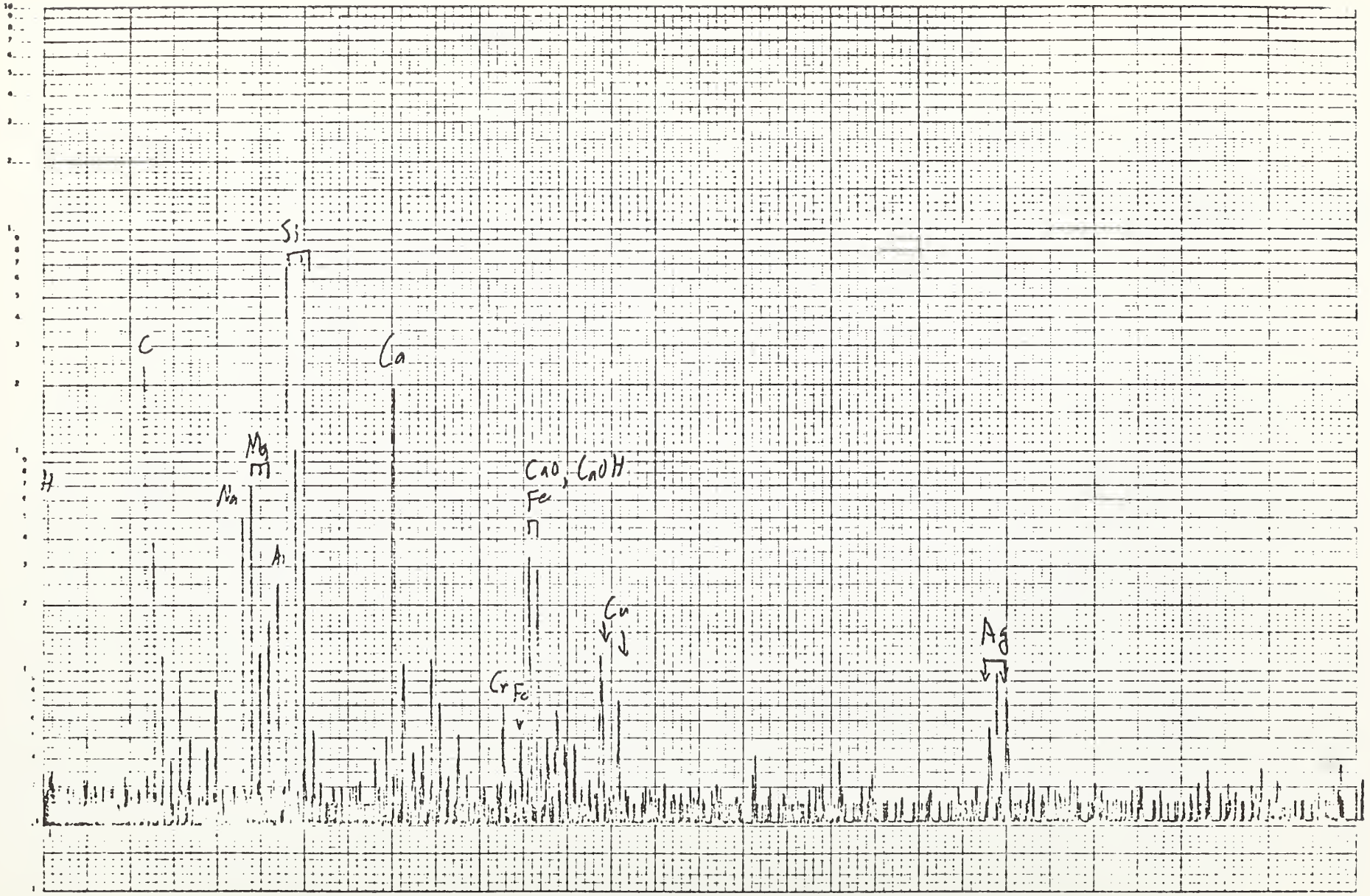

Figure 6. SIMS spectrum of a 200 nm carbon film ion-plated on stainless steel.

CONFORMATION OF ADSORBED γ-GLOBULIN ON SILICON OXIDE

Oxidized Silicon Substrate

In previous Annual Reports to NHLI (1974, 1975), it has been suggested that conformational changes may occur with increasing surface concentration, for bovine γ-globulin and β-lactoglobulin adsorbed on a fused silica surface. Because the results were not entirely conclusive, we have pursued the ellipsometry work in more detail, using as the substrate a specially prepared polished silicon surface covered with a 200 nm film of silicon oxide. This surface was chosen because (1) it is chemically the same as the fused silica previously used in this work, (2) it has superior ellipsometric sensitivity as compared with ordinary fused silica, and (3) it offers the possibility of studying the effects of surface energy on the conformation of adsorbed protein. This last possibility arises because the oxide film, which is hydrophobic, is easily hydrolyzed in hot water, yielding enough SiOH groups to reduce the contact angle of water to zero.

γ-Globulin Purity

Proteins received from commercial sources have sometimes been found to be quite contaminated with impurities, as reported in our Annual Report for 1976. Thus, even though the electrophoretic purity of our commercially prepared γ-globulin was stated by the manufacturer to be 95%, a sample of this protein was fractionated by gel permeation chromatography using Sephadex G-200. The elution chromatogram for this material is shown in Figure 7. There appears to be a small fraction of higher molecular weight (probably dimer) present as a shoulder on the main peak, which was assumed to be monomeric γ-globulin. Fractions 31-40 (mostly dimer) were collected for adsorption experiments in comparison with fractions 50-57 (monomer). Both the adsorbance and molecular extension of the adsorbed "dimer" on hydrophobic

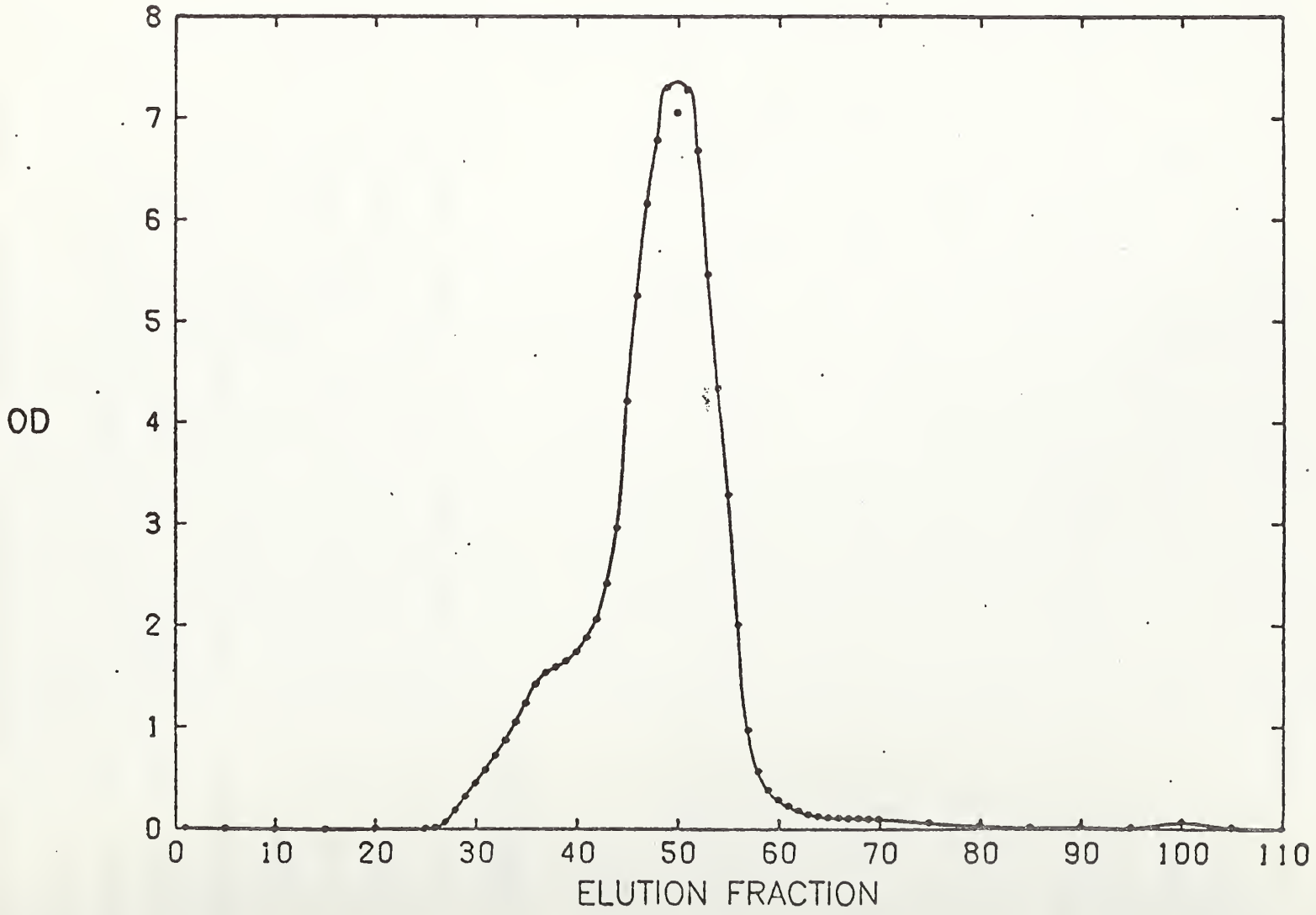

Figure 7. Gel permeation chromatogram of bovine γ-globulin.

silicon oxide were found by ellipsometry to be significantly higher than the values obtained for the monomer. However, the monomer values were the same, within experimental error, as the values obtained for the unfractionated protein. We therefore concluded that it was not necessary to fractionate our γ-globulin in order to obtain meaningful adsorption measurements.

Adsorption on Hydrophilic Silica

Hydrophilic silica was prepared by heating the oxidized silicon wafers in hot water for 20 hours. Thirteen separate γ-globulin adsorption experiments were performed on this substrate with the manual ellipsometer, using a wide range of protein concentrations, from 0.016 to 10.1 mg/ml. The data obtained from these experiments is shown in Figure 8 where the calculated film thickness (molecular extension) is plotted against the calculated adsorbance. Each adsorption experiment yielded 3-5 data points at 1/2 h and 1 h, and at 1 h intervals until the experiment was concluded. All data points are included in the figure. Adsorption from very dilute solution produced low adsorbances and greatly scattered extension values, as seen in the left portion of the plot. The scatter is due to the small differences in refractive index between these very dilute films and the supernatant solution. Clearly, little can be said about the molecular extension in this region. At higher adsorbances, between 2-3 mg/m^2, the data points for individual runs are connected by lines. With only one exception, there is an overall increase in extension with adsorbance within each run, although the magnitude of the increase is variable. The plateau adsorbance, about 3 mg/m^2, is in good agreement with the value 3.4 ± 0.6 previously reported for γ-globulin on fused silica. However, the extension reported on fused silica at the plateau adsorbance was 32 ± 10 nm, which is considerably higher than the average value obtained in this work. Despite the

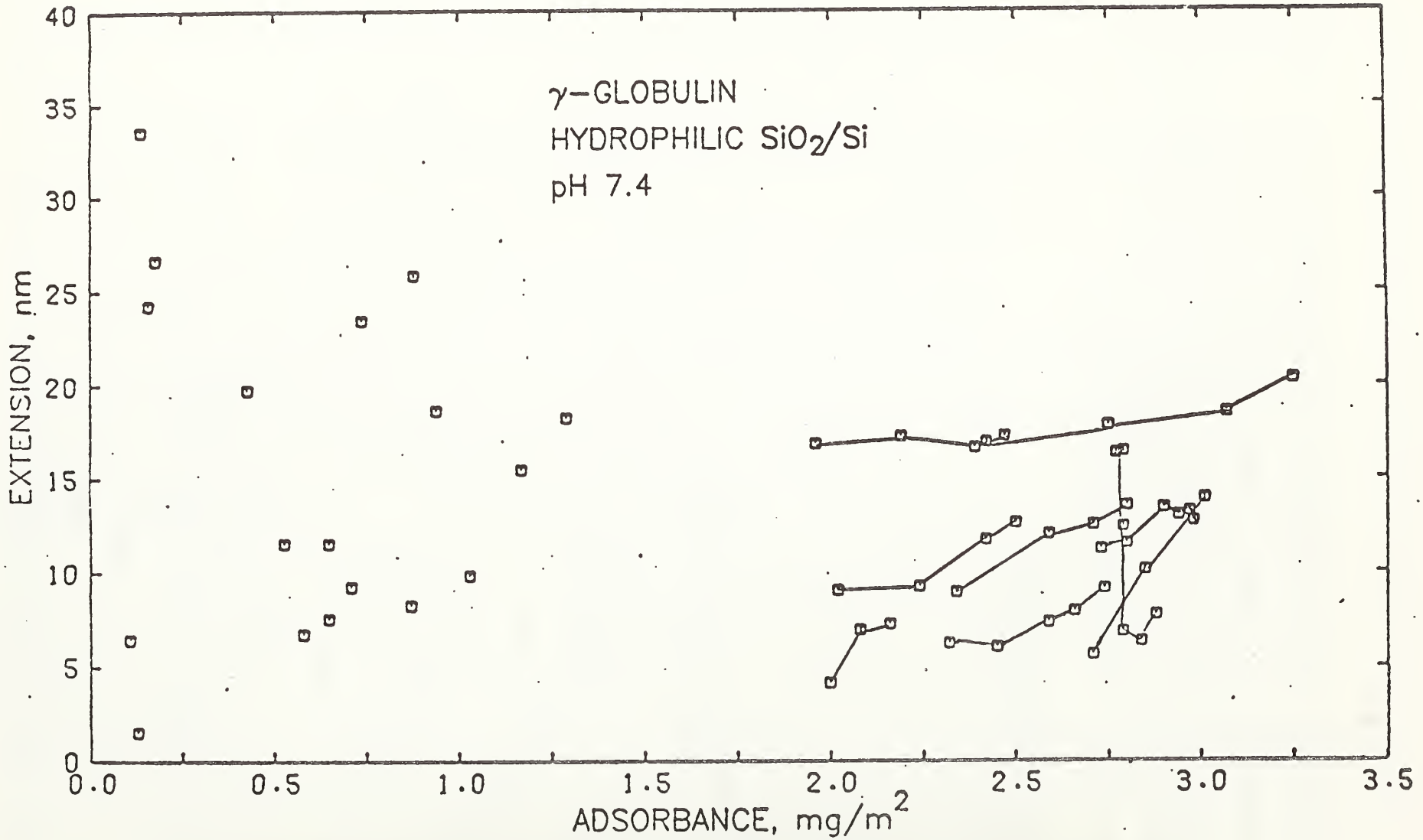

Figure 8. Extension of bovine γ-globulin on hydrophilic silicon oxide, measured with the manual ellipsometer.

apparent increase in extension with adsorbance within individual runs, the average extension does not appear to increase significantly with adsorbance. It is likely that these data are more directly comparable with the fused silica results previously reported than with the following results for the hydrophobic silica, since the fused silica was also hydrophilic.

Adsorption on Hydrophobic Silica

Thirteen separate adsorption experiments were performed on hydrophobic silicon oxide with the manual ellipsometer, and the results are summarized in Figure 9. As compared with the hydrophilic substrate, the plateau adsorbance on the hydrophobic surface is higher by about 1 mg/m^2, and the adsorption isotherm is much steeper. For example, the adsorbance attained on the hydrophobic substrate after 3 h from a concentration of 0.015 mg/ml was 2.8 mg/m^2, whereas a comparable experiment on the hydrophilic substrate produced an adsorbance of only 0.16 mg/m^2. As with the hydrophilic substrate, there was an overall increase in extension with adsorbance within each individual run, although again the magnitude of the increase was not constant.

The results of two adsorption experiments on the hydrophobic surface using the automatic ellipsometer are shown in Figure 10. Very dilute solutions (0.10 and 0.018 mg/ml) were used in these experiments, in order to observe the initial stages of the adsorption process. Maximum probable error bars on three of the data points indicate that the experimental uncertainty in the calculated extension decreases with increasing adsorbance, but is so great at low adsorbances that care must be used in extracting quantitative information. Over the adsorbance range of about 1.2-2.5 mg/m^2, there is no clear trend in the extension with adsorbance.

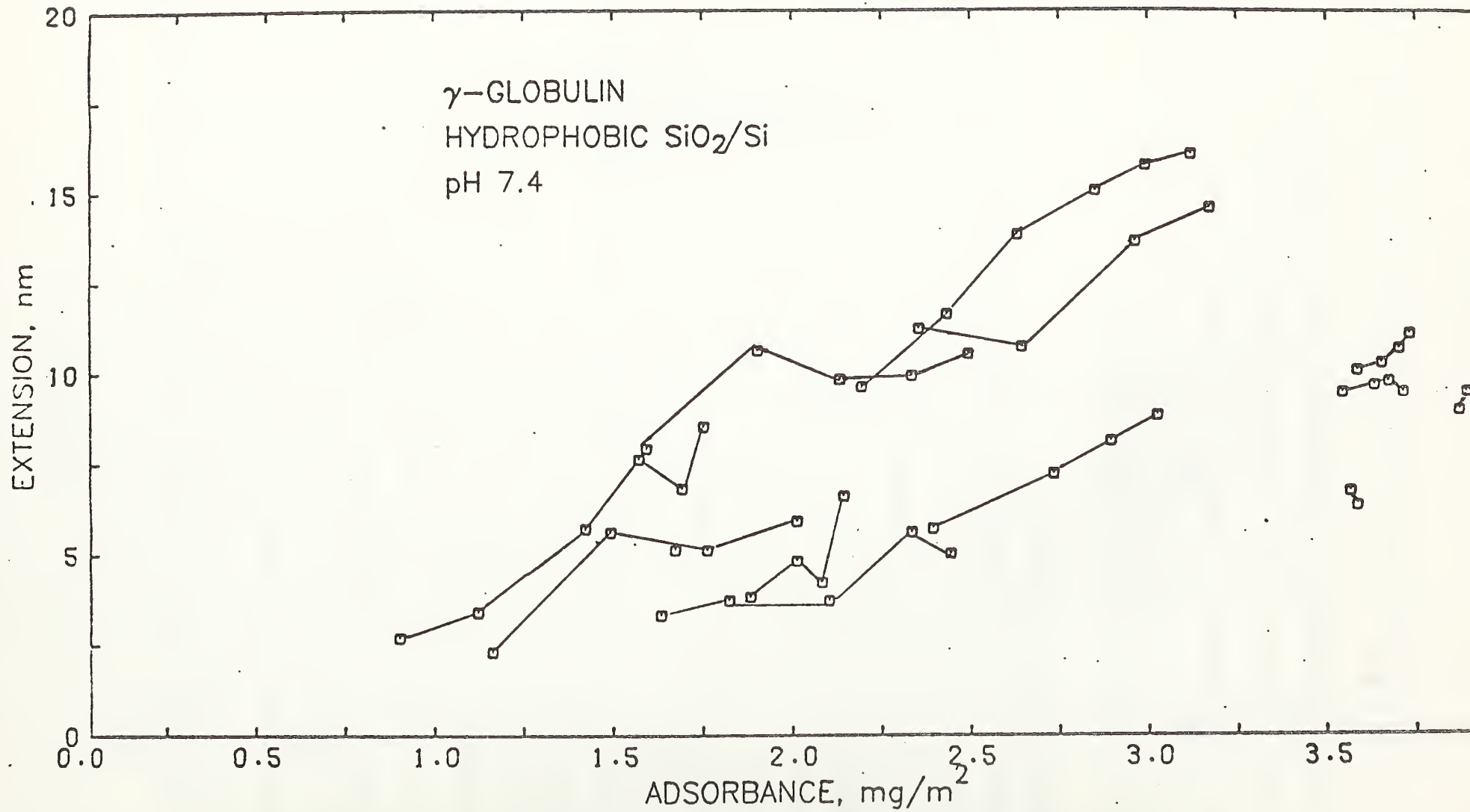

Figure 9. Extension of bovine γ-globulin on hydrophobic silicon oxide, measured with the manual ellipsometer.

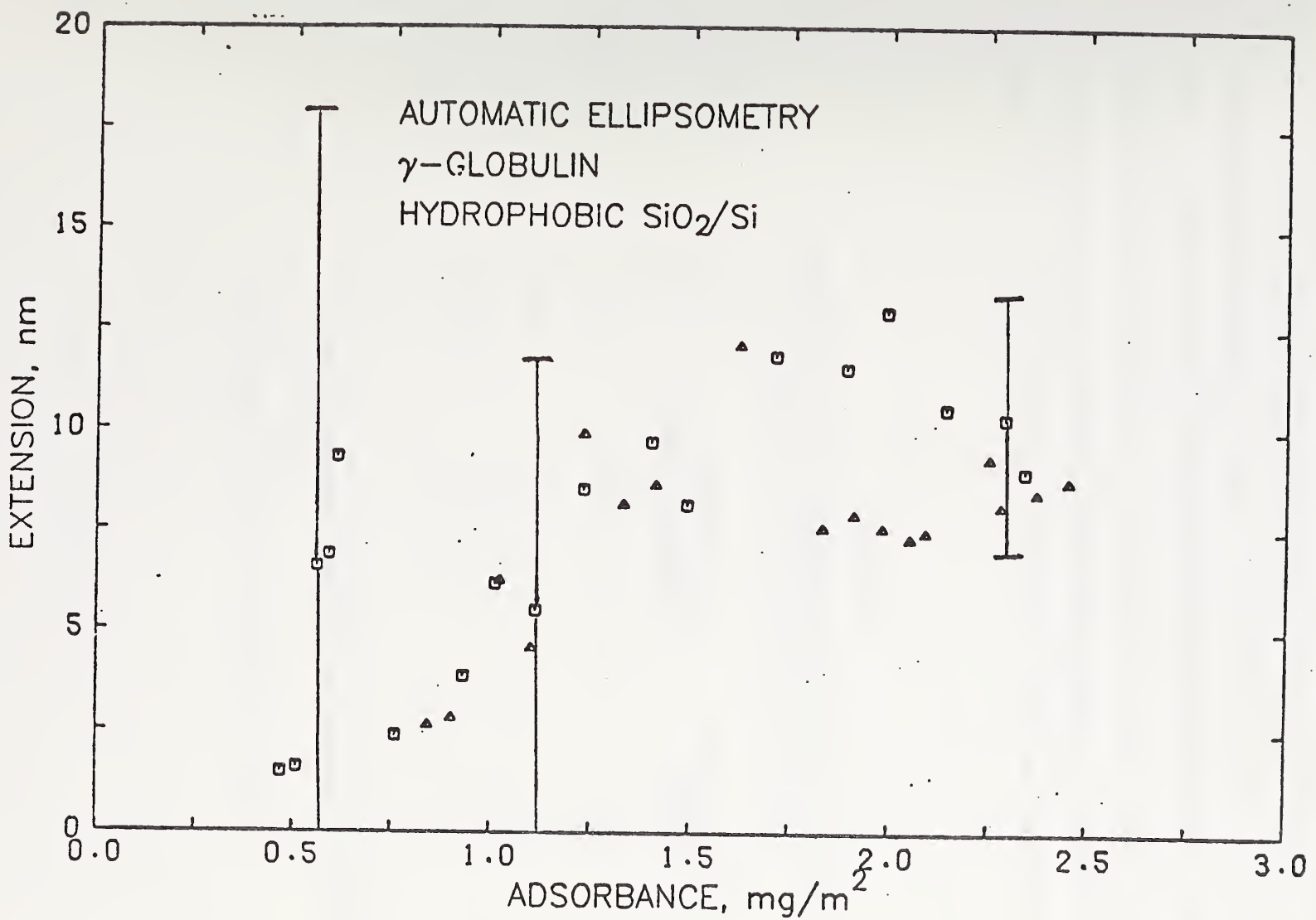

Figure 10. Extension of bovine γ-globulin on hydrophobic silicon oxide, measured with the automatic ellipsometer.

derived from these data, however. It is apparent that little, if any, significant difference exists between the hydrophobic and hydrophilic surface, with respect to their overall net interaction with the protein. Neither the extension nor the adsorbance seems much affected by the surface free energy. However, both extension and adsorbance appear to be significantly lower at the lowest pH, which corresponds to the highest net (positive) molecular charge. It remains to be seen whether a corresponding negative charge produces the same effect. Further work is in progress to expand and replicate these results.

TABLE I

EFFECT OF pH ON ADSORBANCE AND MOLECULAR EXTENSION OF BOVINE γ-GLOBULIN ON OXIDIZED SILICON

pH	Molecular Extension (nm)[a]	Adsorbance[b] (mg/m^2)
3.9	(1) 4.8	2.49
	(2) 5.4	2.18
4.9	(1) 12.6	4.57
	(2) 14.0	3.68
6.4	(1) 13.7	4.17
	(2) 16.0	3.98

(a) (1) is for hydrophobic and (2) is for hydrophilic silicon oxide. Results are average of 1/2 h and 1 h values.

(b) Results are for 1 h adsorption time.

Separation of Aggregates

Before a definitive study of the adsorption of blood proteins on substrates can be undertaken, the degree of purity of these adsorbates must be ascertained. Both labeled and unlabeled proteins have been found by us (6) to contain varying amounts of dimers and higher aggregates as well as low molecular weight polypeptide fragments. Competitive adsorption from such mixtures can lead to apparent protein adsorbances which are quite different from the desired value of the monomer alone.

Commercially available human serum albumin (HSA) has been previously analyzed and separated into fractions by us (6). We have continued to use a gel permeation chromatographic column packed with Sephadex G-200 to separate the various molecular weight components. Figure 11 shows the results for unlabeled HSA (6) fractionation. Portions of the monomer fraction were used to increase the protein concentration of the labeled ^{125}I HSA used for adsorption studies thereby allowing us to conserve valuable labeled protein. The unlabeled sample has less than 0.5% low molecular weight polypeptide, approximately 72% monomer and 28% dimer and higher aggregates. In Figure 12 a similar separation is shown for unlabeled HSA supplied by another commercial source. This material is clearly contaminated with more dimer and higher aggregates. This material was not used in any of our work presented here. In Figure 13 the same separation into fractions is shown for ^{125}I labeled HSA. As shown, a relatively small amount of contaminants are found in this material from supplier (s). The central peak is for the monomer fraction. The other labeled material, HSA(^{131}I), from supplier (n) in Fig. 14 also showed essentially no contamination. However, the ^{3}H HSA, shown in Fig. 15, also from supplier (N) has had little or no contamination by dimer or higher aggregates. There is a small amount of low molecular weight polypeptides present, however.

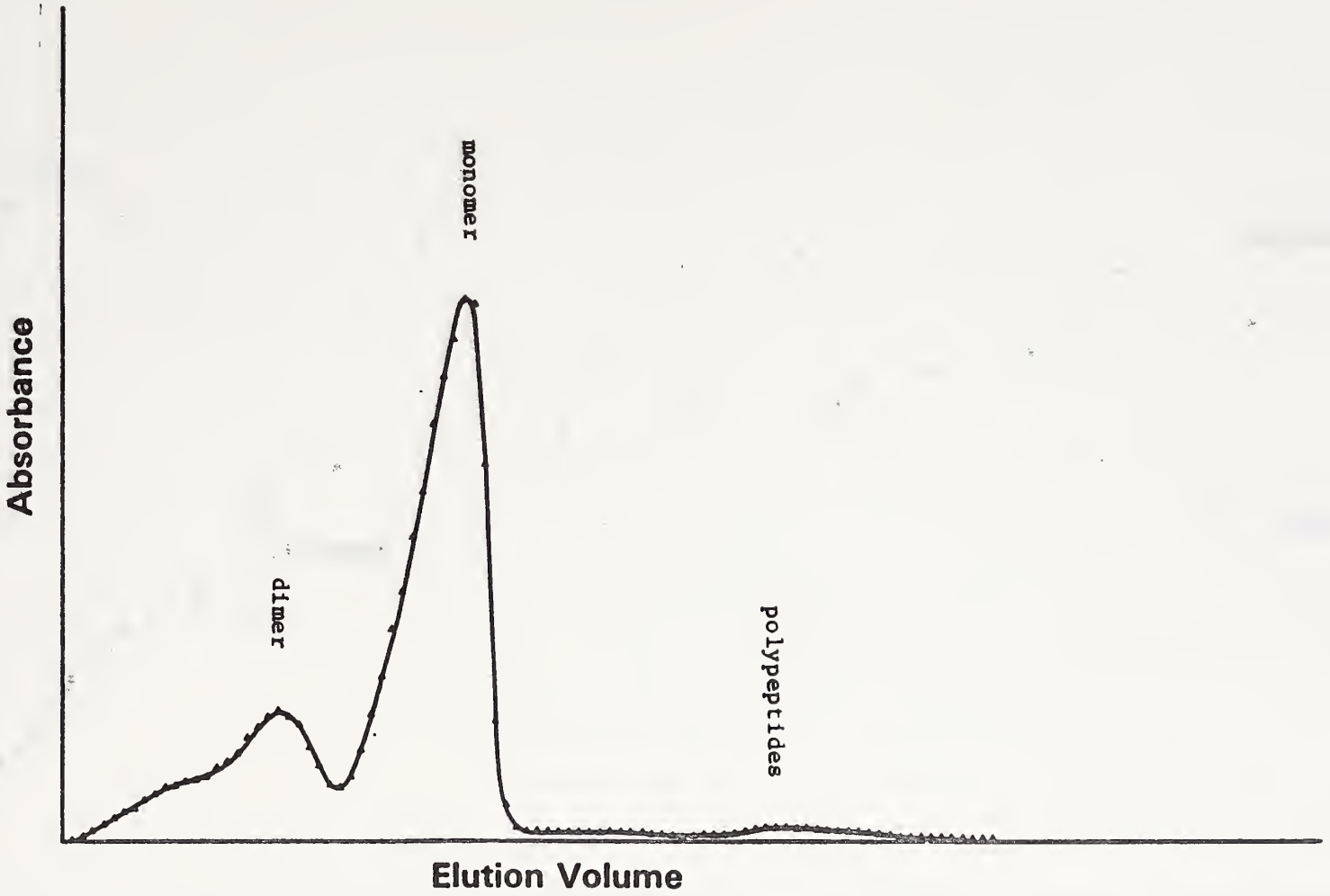

Figure 11. Gel permeation chromatogram of unlabeled HSA.

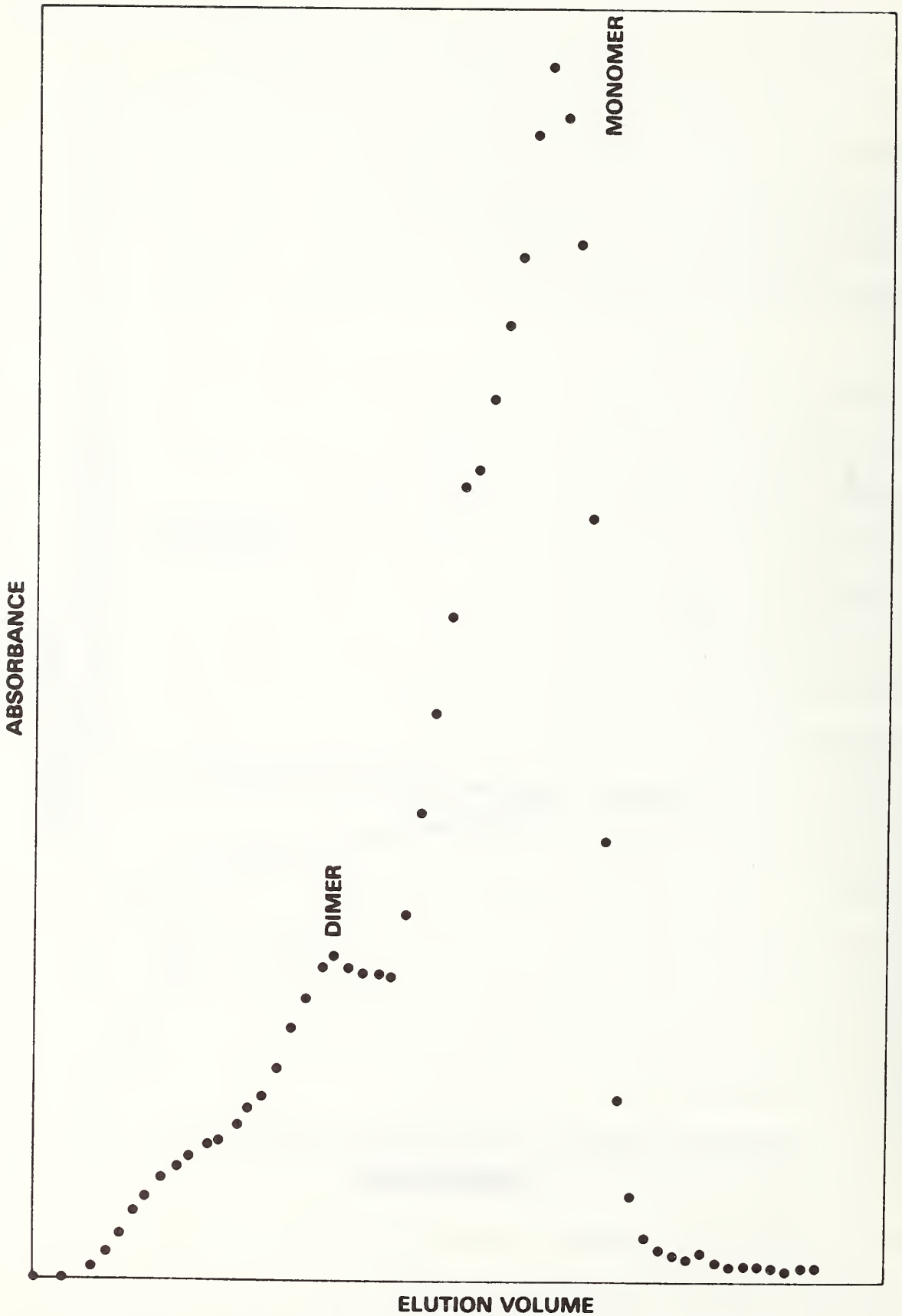

Figure 12. Gel permeation chromatogram of unlabeled HSA.

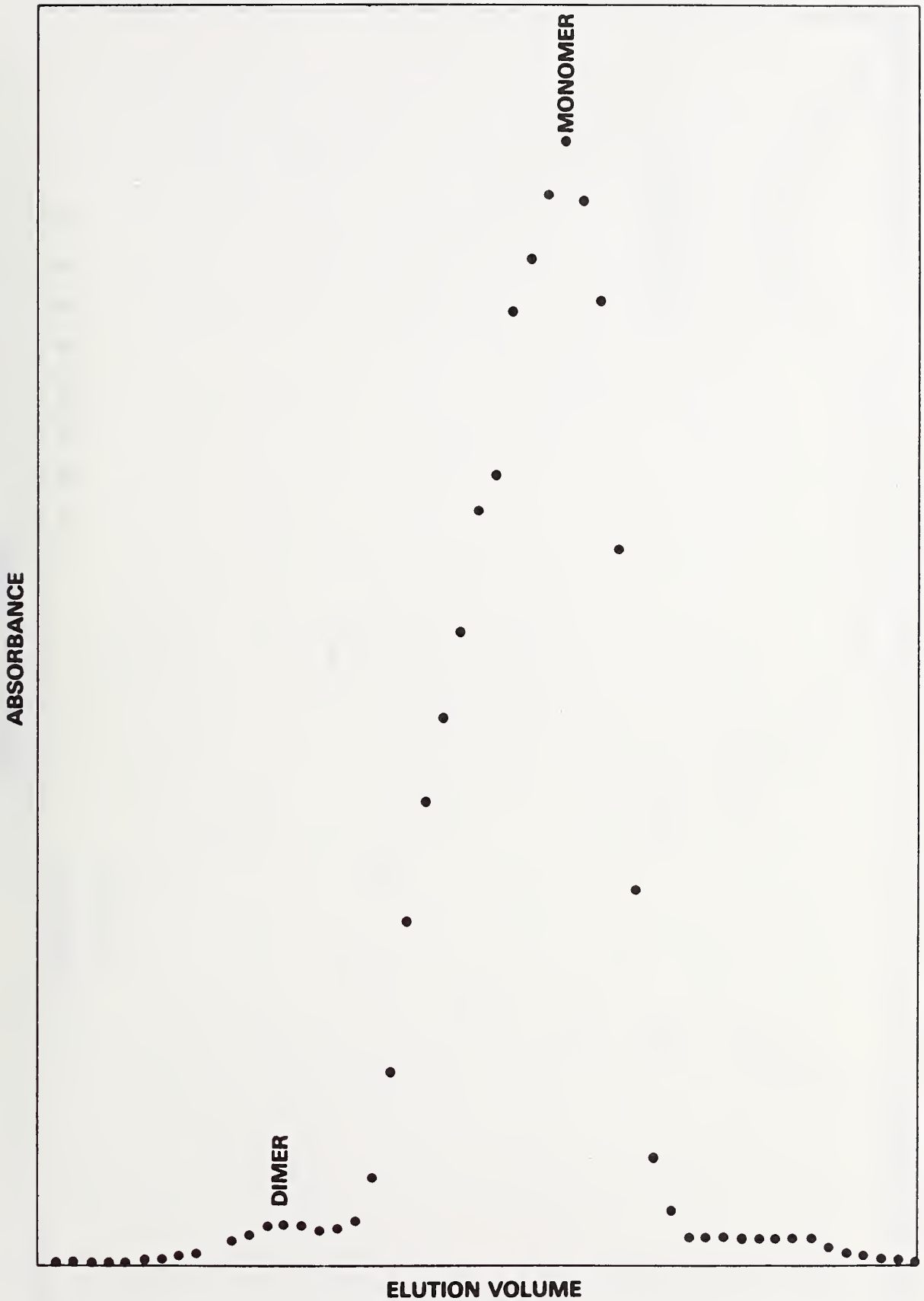

Figure 13. Gel permeation chromatogram of ^{125}I HSA.

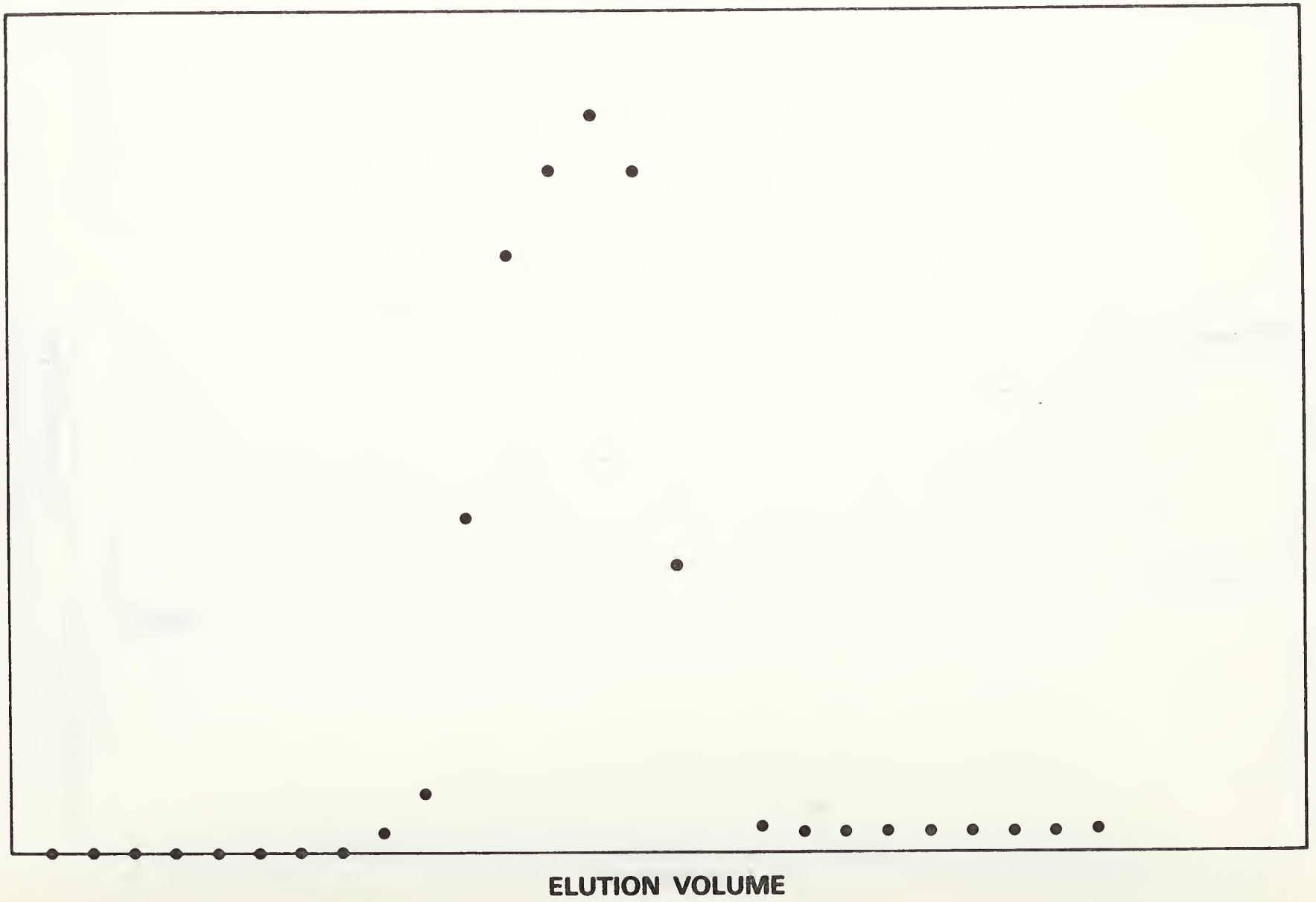

Figure 14. Gel permeation chromatogram of ^{131}I HSA.

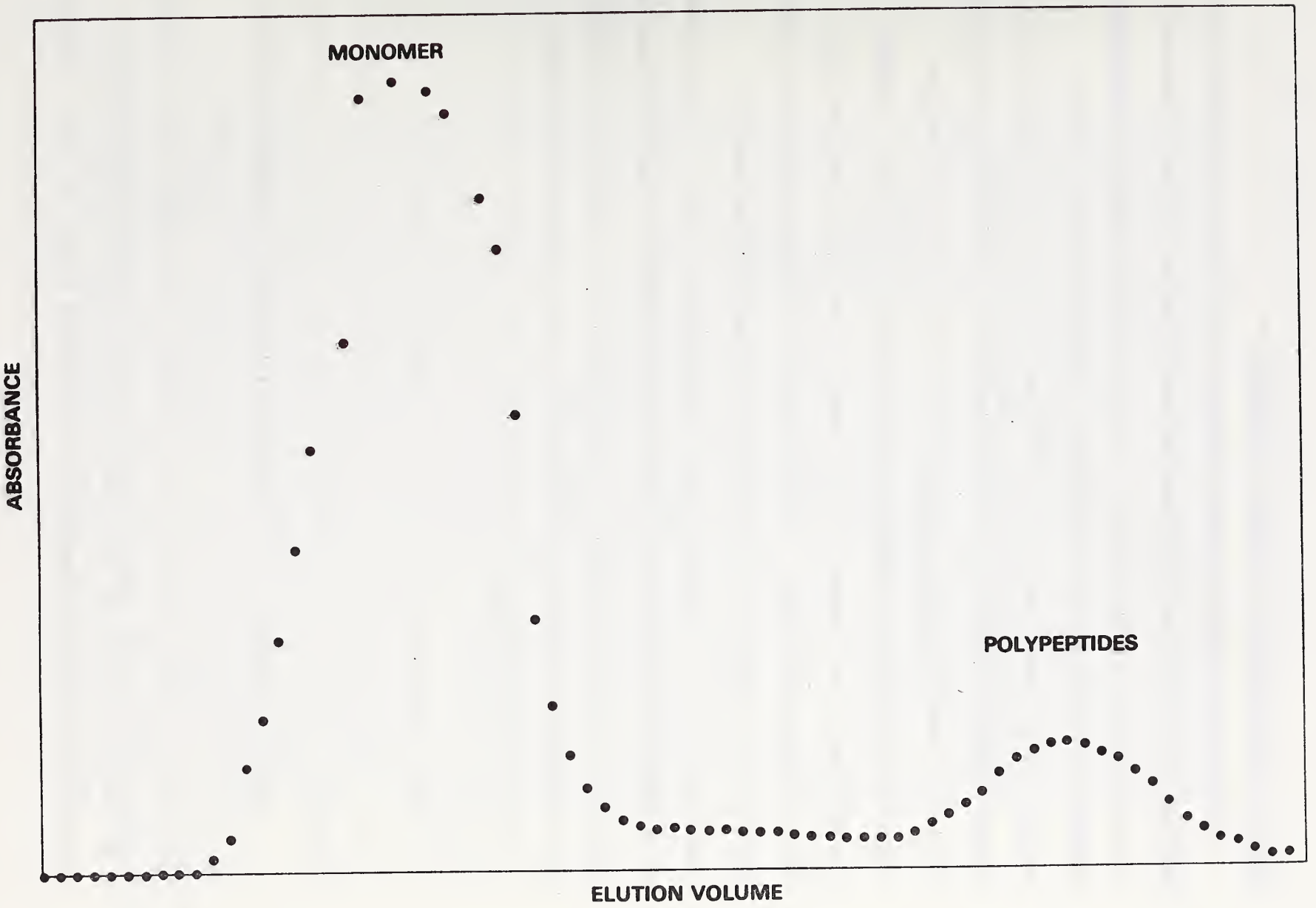

Figure 15. Gel permeation chromatogram of ^{3}H HSA.

Effect of Labeling Atom on Protein Adsorption

Some of the possible errors associated with the use of radiotracers to study protein adsorption have been discussed by us in a recent paper (8). One must consider the possibility that the labeling atom may be specifically adsorbed to a surface, thus enhancing the adsorption of the protein. Isotopes of the same atom, such as ^{125}I and ^{131}I have very different decay schemes, and the energy spectra associated with the decays are quite different. There is a possibility that autoradiolysis resulting from the emitted radiation may lead to an undesirable degradation of the protein, thereby producing molecular fragments which compete for surface adsorption sites with the undegraded protein. The end products of radioactive decay are also different for different isotopes, which introduces further possible complications in the interpretation of the adsorption data. For these reasons, it is necessary to test the effects of various labeling atoms on the adsorptive behavior of labeled compounds. In the present work, we report the effect of two labeling iodine isotopes, ^{125}I and ^{131}I, on the adsorption of human serum albumin on polyethylene.

Linear polyethylene, a well characterized polymer, Standard Reference Material 1475, was obtained from the National Bureau of Standards in the form of solid pellets and molded into flat sheets from which small discs with a surface area of 5.81 cm^2 were cut. The adsorption procedure for this study has been described previously in the Annual Report for 1976. Adsorption

of HSA(^{125}I) (7.66 mg/ml) was carried out for 30 minutes, 1 h, 2h and 3h and the adsorption of HSA(^{131}I) for a time period of only 1 h followed by our standard quick rinse, drying and counting procedure. The adsorption results are given in Table II.

TABLE II

ADSORPTION OF HSA(^{125}I) AND HSA(^{131}I) ON POLYETHYLENE

Adsorption Time	Adsorbance (mg/mg^2) HSA(^{125}I)	HSA(^{131}I)
30 min	3.58	
1 hour	4.68	3.71
2 hour	5.00	
3 hour	5.04	

Initially, for the adsorption of HSA(^{125}I), as observed in Table II there is an increase in the adsorbance between 30 min and 1 h, with little change between 1h and 3h. The small difference in the 1 h results for the two isotopes is probably due to the difference in concentration of the solutions. The rate of adsorption, and perhaps the equilibrium adsorbance as well, would likely be somewhat less for the less concentrated HSA(^{131}I) solution, which is in qualitative accord with the observed results. From these data, we conclude that there is apparently no significant difference in the adsorbance of HSA tagged with the different iodine isotopes.

We are now about to find out how the iodine label compares with a ^{3}H label, in its effect on HSA adsorbance. The ^{3}H labeled HSA has been fractionated by chromatography, according to our usual procedure, and the elution chromatogram is shown in Fig. 15. The fractionated protein will be used to study adsorbance on polyethylene and other surfaces.

DISTRIBUTION OF ADSORBED PROTEINS ON SURFACES

Autoradiographic measurements of HSA(^{125}I) adsorption have been performed on a variety of surfaces, some of which have been noted in earlier sections of this report. It is clear that this technique can yield much valuable information about the distribution of adsorbed proteins, as well as semiquantitative information about the total amount adsorbed. The other surfaces which we have thus far examined by this technique are polyethylene, glass, oxidized silicon, and platinum. Two week autoradiographic exposures were clearly not sufficient for the first 3 of these substrates. Additional exposure time and/or the use of higher-activity labels will be necessary in order to derive useable information about these surfaces. The platinum surface, however, has shown some interesting and unexpected patterns of protein adsorption, as we will show.

Figs. 16 through 19 are the autoradiographs of Pt slides, which were subjected to different surface treatments. Fig. 16 shows a very intricate four-fold symmetry pattern in its adsorption of HSA(^{125}I). The surface was treated only by acid cleaning and rinsing. While the exact cause of this adsorption pattern is not understood as yet, it could result from stress-induced crystallization or microcrack formation propagated by the shearing of the metal plate, producing high energy sites on the surface and leading to enhanced protein adsorption. Since the presence of high energy sites on biomaterials is often postulated as a cause of enhanced adsorption of blood proteins, the opportunity to test this idea, using a material on which the surface energy may be altered by the introduction or removal of high energy sites is intriguing and potentially of considerable use in understanding the mechanism of protein adsorption.

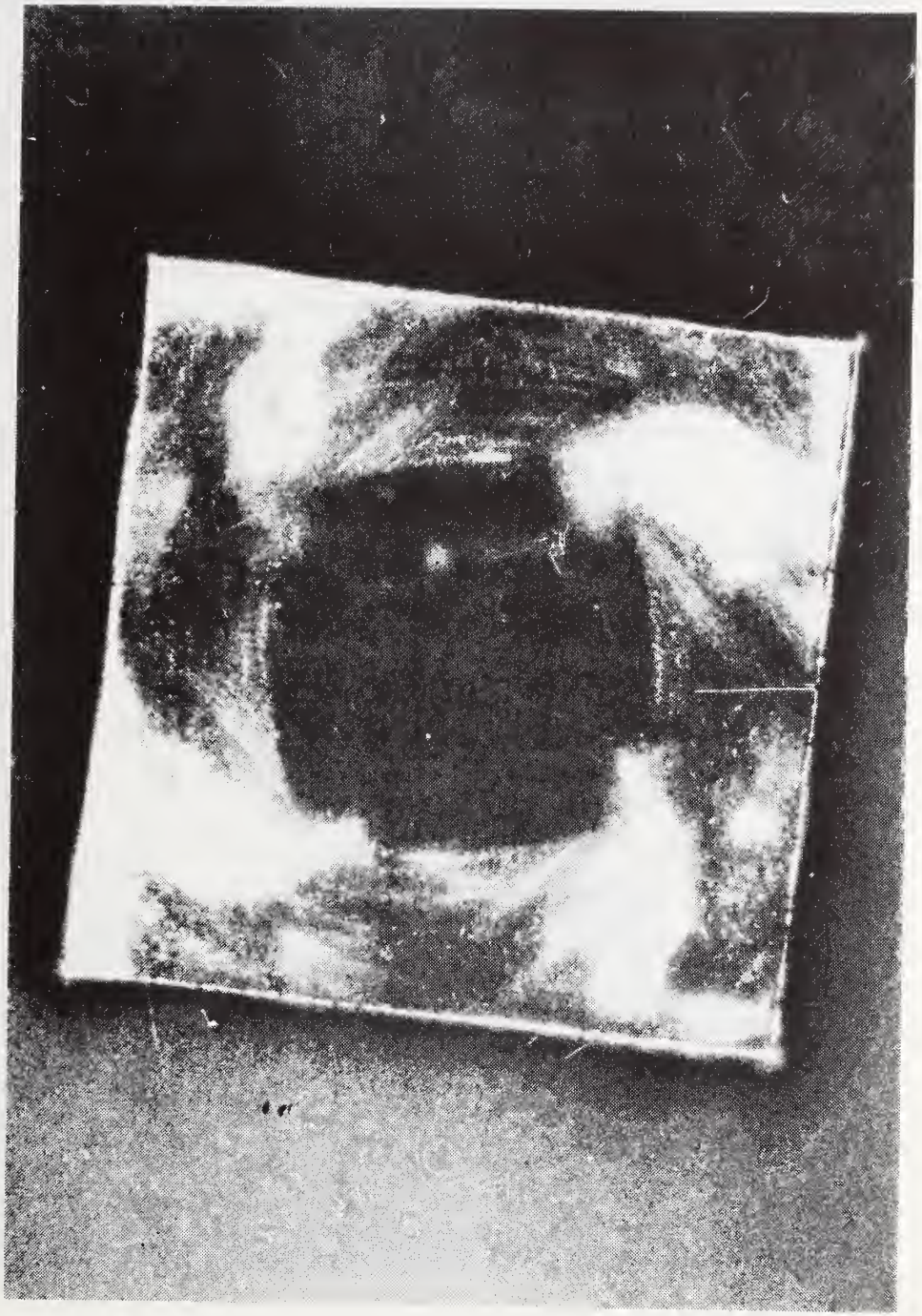

Figure 16. Autoradiograph of Pt slide with adsorbed HSA(^{125}I). Acid cleaned only.

Fig. 17 is the autoradiograph of another Pt slide, which was acid-cleaned and heated in a muffle furnace at 500°C for 10 min. Enhanced adsorption is apparent in the mosaic light pattern, which suggests the presence of high-energy sites at grain boundaries between metal crystals. We will pursue this line of investigation, to determine by surface etching and other techniques whether the enhanced protein adsorption can be correlated with known physical properties of the metal surface.

Figs. 18 and 19 are the autoradiographs of the two sides of the same Pt slide, which was acid-cleaned, heated at 500°C for 10 min, and flamed to red heat for 20-30 s, prior to protein adsorption. As in Fig. 16, mosaic patterns are seen, but the areas of enhanced adsorption are much larger, suggesting the possibility of metal crystal growth during the intense heat treatment. These ideas will require further testing and corroboration by other physical means.

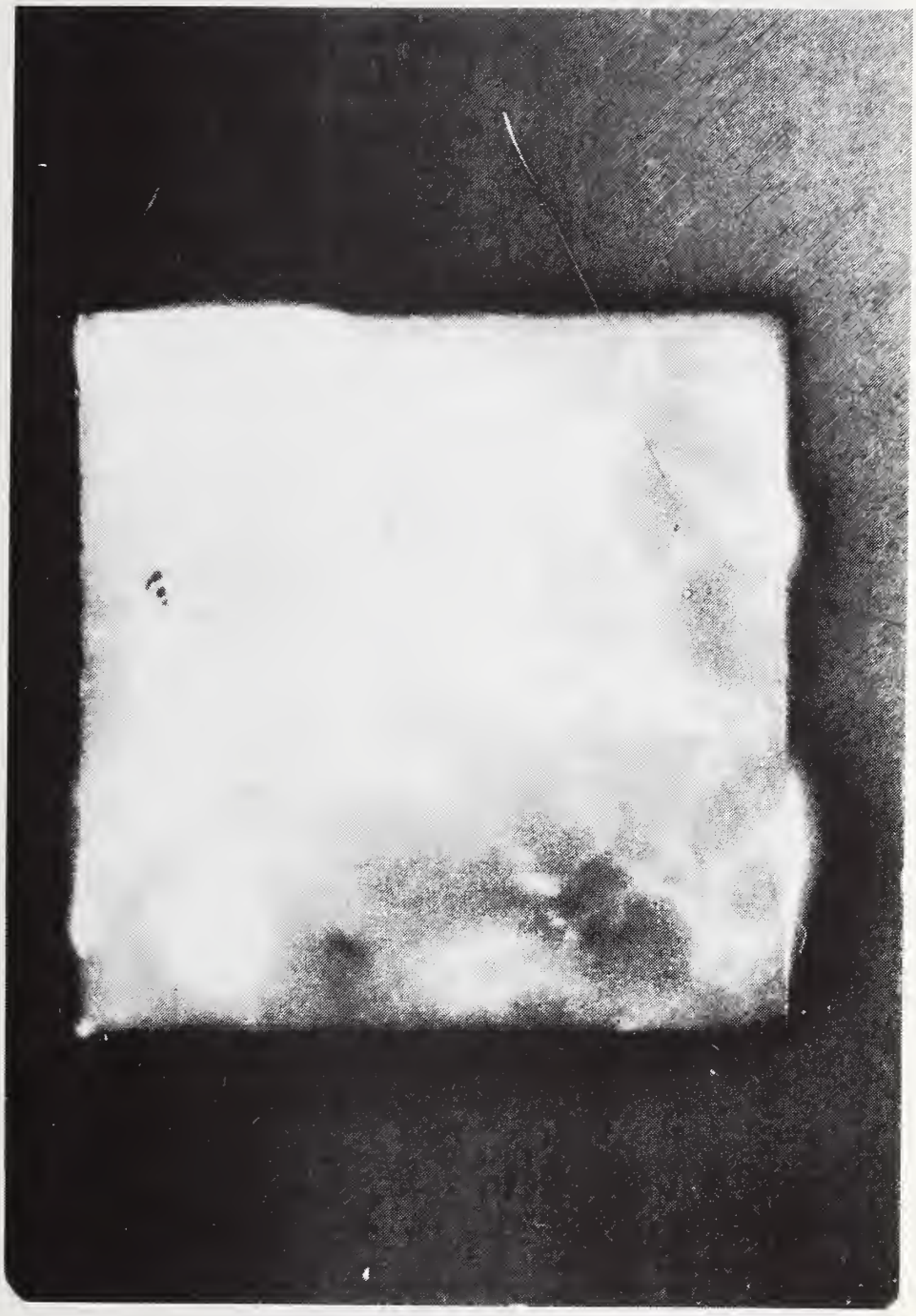

Figure 17. Autoradiograph of Pt slide with adsorbed HSA(^{125}I). Surface was acid cleaned and heated to 500°C.

Figure 18. Autoradiograph of Pt slide with adsorbed HSA(^{125}I). Surface acid cleaned and flamed to red heat.

Figure 19. Autoradiograph of Pt slide with adsorbed HSA(^{125}I). Surface acid cleaned and flamed to red heat.

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16. Abstracts

The purpose of this investigation is to help develop methods for characterizing the surface properties of cardiovascular implant materials. It is expected that these properties, which are related to the success or failure of implants, will ultimately form the basis for in vitro testing of prospective implant materials. The investigation is focused upon three aspects of blood protein adsorption on surfaces, namely (1) the total amount of protein adsorbed, (2) the spatial distribution of adsorbed protein on the surface, and (3) the conformation of adsorbed protein molecules. A number of materials produced by contractors to the NHLBI Biomaterials Program have been examined by ellipsometry and by the adsorption of radiolabeled proteins. Autoradiography has been used to study the uniformity of protein adsorption on several surfaces. Evidence for the migration of proteins into biomaterials has been discovered. Adsorption studies of γ-globulin on a specially prepared oxidized silicon surface have contributed to our basis understanding of the adsorption of blood proteins on surfaces.

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- [ ] CONTROLS
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