THE THICKNESSES OF HEMOGLOBIN AND BOVINE SERUM ALBUMIN MOLECULES AS UNIMOLECULAR LAYERS ADSORBED ONTO FILMS OF BARIUM STEARATE*

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The following work, which describes a method of measuring one dimension of some protein molecules, is based on the determination of the apparent thickness of a unimolecular layer of globular protein molecules adsorbed from solution onto a metallic slide covered with an optical gauge of barium stearate. Langmuir[1, 2] and Rothen[3] have published a few results obtained by such a technique, but have not exploited the method thoroughly. A complete set of experimental data has been obtained by Clowes[4] on insulin and protamine. He studied the effects of *p*H and time of exposure on the thickness of layers of protamine and insulin adsorbed onto slides covered with barium stearate and conditioned with uranyl acetate. He found that the *p*H was responsible for large variations in the thickness of the adsorbed layers and that the thickness of insulin layers adsorbed onto a protamine base was dependent on the concentration of the insulin. Since Clowes found thicknesses as high as 100 Å. for protamine and 400 Å. for insulin, he was without doubt usually dealing with multilayers.

Experimental.—The apparent thickness of a protein layer is measured with an optical instrument called the ellipsometer by Rothen,[5–7] who has given a complete description of its design and optics and has calculated its sensitivity as 0.3 Å. This instrument measures the ellipticity of light reflected from a metallic slide when it is covered with a thin film of transparent material. The parameters of the ellipse of polarization are determined in part by the thickness and refractive index of the transparent layer of material on the slide. The ellipsometer, which is a type of polarimeter, uses the half-shadow technique, by which a change in the ellipticity of the reflected light requires a change in angular setting of an analyzer used to balance the intensity of the half fields. As actually used, the instrument is calibrated with films of barium stearate, which for different known thicknesses, previously determined by x-ray diffraction measurements, require different angular settings of the analyzer for equal intensity of the half fields. In this way one measures the angular change in the analyzer setting produced by a film of unknown thickness and relates it by a calibration curve to a known thickness of barium stearate.

Such a calibration assumes that the indices of refraction of films of

barium stearate and globular proteins are the same. The indices of refraction of hemoglobins and serum albumins in the unhydrated state have been calculated by Putzeys and Brosteaux[8] and by Armstrong[9] to be 1.60. Using data obtained by Bull[10] on the hydration of proteins in equilibrium with various partial pressures of water, one can correct the refractive index of 1.60 for hydration of the dry films resulting from the relative humidity of the laboratory. By using the Lorentz-Lorenz equation for such a mixture of protein and water, one obtains an effective refractive index of 1.57. However, it is reasonable to suppose that the globular protein molecules do not occupy the entire surface of the barium stearate, but pack together in such a way as to leave voids between themselves. This condition will further reduce the refractive index, which will be dependent on the type of packing assumed for the adsorbed protein molecules. It has been assumed that when the barium stearate surface is saturated with a monolayer of protein molecules the packing may be best approximated by the closest packing of elliptical cylinders resting on their bases. The fractional volume occupied by such a molecular model is 0.91, and if one assumes that the voids are occupied by air the effective refractive index, calculated by the Lorentz-Lorenz equation for a mixture, is 1.50. Since the refractive index of a barium stearate film is 1.50, the apparent thickness of a film of globular protein molecules, if one makes the above assumptions, is optically equivalent to the thickness of a film of barium stearate. Other assumptions concerning the degree of hydration and the percentage of voids would change the apparent measured thickness of the protein molecules by 1 or 2 Å.

The general technique used in these experiments was similar to that used by Rothen[3] in his work on antigen-antibody reactions. With the Blodgett and Langmuir[11] methods an optical gauge of barium stearate was placed on highly polished stainless steel slides which had been thoroughly cleaned with Shamva, a metallographic polish. The stearic acid, dissolved in redistilled benzene, was spread on redistilled water. The zero point reading was then determined on the ellipsometer. The carbonmonoxyhemoglobin solutions were prepared from crystalline carbonmonoxyhemoglobin preparations obtained by the method of Drabkin,[12] and all dilutions were done with 0.003 *M* potassium phosphate buffer at *p*H 6.8. Concentrations of carbonmonoxyhemoglobin were determined colorimetrically on a Klett colorimeter, which had been calibrated by Kjeldahl analyses for nitrogen. The crystalline bovine serum albumin, obtained from Armour's Research Laboratories, was dissolved in acetate buffer of ionic strength 0.15 and *p*H 4.9.

For the adsorption it was found that the most reproducible results were obtained by placing the slides covered with barium stearate for five minutes in 5-ml. beakers containing the protein solutions. The slides were then

thoroughly washed in running distilled water for five minutes. For constant results a thorough and reproducible washing procedure was found to be of importance. After washing, the slides were allowed to dry in air, and the thicknesses of the adsorbed protein layers were then measured on the ellipsometer. All solutions and wash water were maintained between 16 and 19°C. The error for any one set of measurements was about 10%.

Results.—The results may be seen in Table 1 and Figure 1.

TABLE 1

THE APPARENT THICKNESSES OF SOME PROTEIN FILMS AT VARIOUS CONCENTRATIONS OF PROTEIN

CONCENTRATION, G./100 ML.	HUMAN CARBON-MONOXYHEMOGLOBIN, *d* IN Å.	HORSE CARBON-MONOXYHEMOGLOBIN, *d* IN Å.	BOVINE SERUM ALBUMIN, *d* IN Å.
5	37	..	35
4	39	..	..
3	38	37	34
2	39	34	..
1	37	34	32
0.8	35	40	..
0.6	32	36	..
0.5	..	..	27
0.4	37	32	..
0.2	30	29	..
0.1	25	36	25
4×10^{-2}	19	..	..
2×10^{-2}	..	39	23
8×10^{-3}	12	..	..
1.6×10^{-3}	13	..	..
8×10^{-4}	..	33	..
2×10^{-4}	..	..	10
8×10^{-6}	..	35	..
5.3×10^{-6}	..	25	..
2×10^{-6}	..	..	0
5.3×10^{-8}	..	25	..

Interpretation of Results.—The data in Table 1 have been interpreted by using the simple Langmuir adsorption equation. If one assumes that the apparent thickness of the film, d, is proportional to the fraction of the surface covered, one obtains as the equation for the adsorption of a unimolecular layer of protein molecules onto a solid surface

$$C/d = 1/bd_m + C/d_m,$$

where

C = concentration of protein in g./100 ml.,
d = apparent thickness of the film,

d_m = the apparent thickness of a unimolecular layer of close-packed protein molecules, and

b = a constant related to the heat of adsorption.

It is seen that a plot of C/d against C should give a straight line, and that the reciprocal of the slope of this line is the apparent thickness of a unimolecular layer of protein molecules. Figure 1 shows that a straight line was obtained, and the reciprocals of the slopes of these lines give the thickness of the horse carbonmonoxyhemoglobin molecule as 36 Å., the human carbonmonoxyhemoglobin molecule as 38 Å., and the bovine serum albumin molecule as 34 Å.

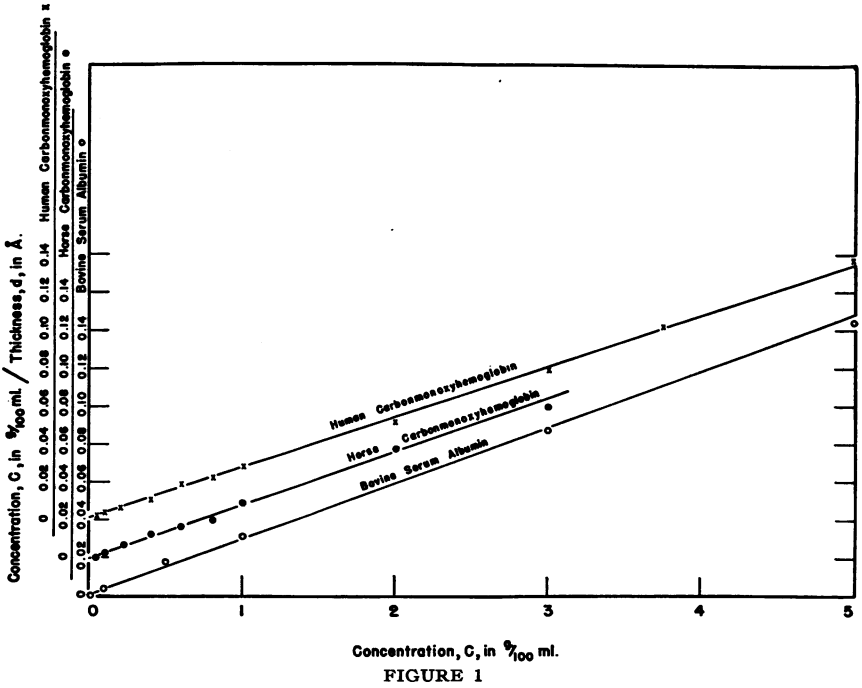

FIGURE 1

Langmuir adsorption isotherms for proteins adsorbed onto barium stearate, t = 16–19°C.

In one respect the conditions of our experiments departed from those assumed in the derivation of the adsorption isotherm equation; namely, that an equilibrium does exist between the molecules in solution and the adsorbed molecules. Our washing procedure amounted to placing the slide in an infinitely dilute solution, and one would therefore expect that, given enough time, all molecules would be desorbed. Our results indicate

that the five-minute period of washing was sufficient to remove any multilayers of hemoglobin adsorbed onto the initial layer, but did not appreciably affect the number of molecules adsorbed onto the barium stearate. That the rate of desorption can be very slow is seen in the horse carbonmonoxyhemoglobin experiments, where concentrations of 8×10^{-6} g./100 ml. or 10^{-9} molar apparently still gave unimolecular layers.

A second factor to consider is the hypothesis that the apparent thickness of the film, d, is proportional to the fraction of the surface covered. The Lorentz-Lorenz equation for a mixture of protein and air may be written

$$(n^2 - 1)/(n^2 + 2) = fC_n,$$

where

n = refractive index of the mixture,
$C_n = (n_p^2 - 1)/(n_p^2 + 2)$,
n_p = refractive index of the protein, and
f = fraction of the surface covered.

Using the Drude equation, $\Delta = -A(1 - 1/n^2)d_m$, for the phase difference, Δ, between the components of the ellipse of polarization resulting from a film of actual thickness, d_m, and substituting for n, one obtains

$$\Delta = \frac{-3Ad_m}{2 + 1/(fC_n)},$$

where A is a constant. Since Rothen[7] has found that Δ is mainly responsible for the apparent thickness of the film, d, and a plot of the above equation in the region of physical significance shows that the relation between Δ and f is approximate proportionality, one is probably justified in using the Langmuir equation.

The value of 36 Å. for the thickness of the horse carbonmonoxyhemoglobin molecule may be compared with the value, 34 Å., obtained by Perutz[13] for the $c \sin \beta$ dimension in his x-ray diffraction work on horse ferrihemoglobin. It is believed that the close agreement between the two values, obtained independently, is significant.

The value of 34 Å. for the thickness of the bovine serum albumin molecule is to be compared with the value 40 Å. resulting from measurements on the double refraction of flow of bovine serum albumin made by Edsall and Foster[14] and with the value 38 Å. for the human serum albumin molecule obtained by Oncley, Scatchard and Brown.[15] Since the 40 Å. and 38 Å. values are for the minor axes of prolate ellipsoids of revolution, one would expect our value of 34 Å., which is an average thickness value, to be less.

The unimolecular layer method of determining one dimension of a globular protein molecule can presumably be applied to other proteins. In conjunction with the other methods of measurement it should prove

helpful in providing information about the size and shape of protein molecules.

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Summary.—By use of an optical method it has been found that the thickness of a unimolecular layer of human carbonmonoxyhemoglobin molecules adsorbed onto a film of barium stearate is 38 Å., that of horse carbonmonoxyhemoglobin molecules is 36 Å., and that of bovine serum albumin molecules is 34 Å.

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