Fluorescein Binding to Normal Human Serum Proteins Demonstrated by Equilibrium Dialysis

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● **The binding of fluorescein to normal human serum proteins in a physiologic solvent at 37 °C was measured by equilibrium dialysis. Human serum contained 3.28×10^{-3}M concentration fluorescein-binding sites, with an average association constant at 37 °C of 0.54×10^{4}M^{-1}. The percentage of total fluorescein bound by human serum proteins ranged from 83% to 53% when the total fluorescein concentration ranged from 6.9×10^{-5} to 6.2×10^{-3}M.**

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Fluorescein has been widely used as a tracer to test the permeability of the blood-ocular barrier for two decades.[1,2] Although several studies have been carried out on the binding of fluorescein to isolated serum albumin, whether fluorescein is substantially bound to any plasma protein after intravenous injection in man remains controversial.[2-7]

Laurence[5] initially demonstrated that isolated bovine serum albumin showed concentration-dependent binding of fluorescein by fluorescence polarization. He also reported that the intensity of fluorescein fluorescence dropped markedly concomitant with binding to bovine albumin (fluorescence quenching), and the fluorescein absorption spectrum was red shifted.[5] Both observations are further evidence that fluorescein does bind to bovine albumin. Andersson et al[6] subsequently demonstrated fluorescein binding to isolated bovine albumin by equilibrium dialysis at 5 to 30 °C.

Recently, however, Ianacone et al[7] have studied the binding of radioactive fluorescein using polyacrylamide gel electrophoresis and gel filtration and have questioned whether fluorescein is bound in significant quantity to any plasma protein under physiologic conditions.

A knowledge of the character of fluorescein binding to blood proteins under physiologic conditions is important for an understanding of quantitative fluorescence measurements, both in clinical diagnosis and in experimental research. We therefore have reexamined this question using equilibrium dialysis, a classic method firmly established on thermodynamic theory that is well suited to study small ligand binding to proteins.[8-14] Fluorescein binding to human serum proteins at 37 °C in a physiologic solvent was measured instead of binding to isolated bovine albumin at low temperatures. Substantial concentration-dependent binding of low affinity was observed throughout a wide range of fluorescein concentrations.

MATERIALS AND METHODS

Fresh blood was obtained from normal male volunteers. Serum was used immediately or stored at 4 °C until use. Serum proteins were diluted tenfold with Tyrode's solution (pH 7.4) for equilibrium dialysis.

Fluorescein sodium (Funduscein) was diluted with Tyrode's solution to give concentrations of 12.5 to 2,500 mg/L (0.33×10^{-4} to 0.66×10^{-2}M).

Equilibrium dialysis was performed with specially designed cells, each of which comprised two identical closed compartments separated by a 23-mm (diameter) cellulose-disk dialysis membrane.[10,13] The cells were immersed in a constant-temperature water bath at 37 °C and rotated at a speed of 5 rpm. Three groups of experiments were carried out. In group 1, a given concentration of fluorescein solution (eg, 12.5 mg/L) was dialyzed against Tyrode's solution for variations of dialysis time from 15 minutes to 50 hours to determine the time required to attain equilibrium of ligand diffusion across the dialysis membrane. In group 2, a series of concentrations of fluorescein were dialyzed against Tyrode's solution alone to determine the amount of dye bound by the cellulose membrane as a function of the free fluorescein concentration at equilibrium. In group 3, the series of concentrations of fluorescein solutions were dialyzed against the 1:10 diluted serum. The dialysis was terminated after 50 hours in experimental groups 2 and 3. All cells in the three groups were run in duplicate. The optical density of the fluorescein in the nonprotein compartment was measured at λ 490 nm with a spectrophotometer (Zeiss PMQ II) using a 10-mm path-length cell. The molar extinction coefficient (ε) of fluorescein at 490 nm was determined to be 8.6×10^{4}. The data of the group 3 experiments (dialysis of fluorescein against serum proteins) were corrected for ligand binding by the cellulose dialysis membranes with the data of experimental group 2. Protein-bound

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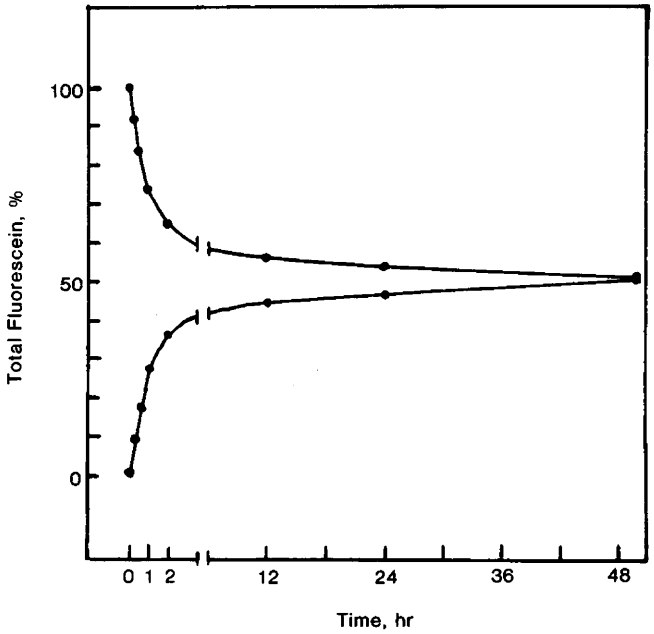


Fig 1.—Time required to attain equilibrium of fluorescein diffusion across dialysis membrane in absence of serum proteins. Percent of total fluorescein present in two compartments of equilibrium dialysis cell (upper curve, initial fluorescein solution side; lower curve, initial solvent side) is plotted against dialysis time.

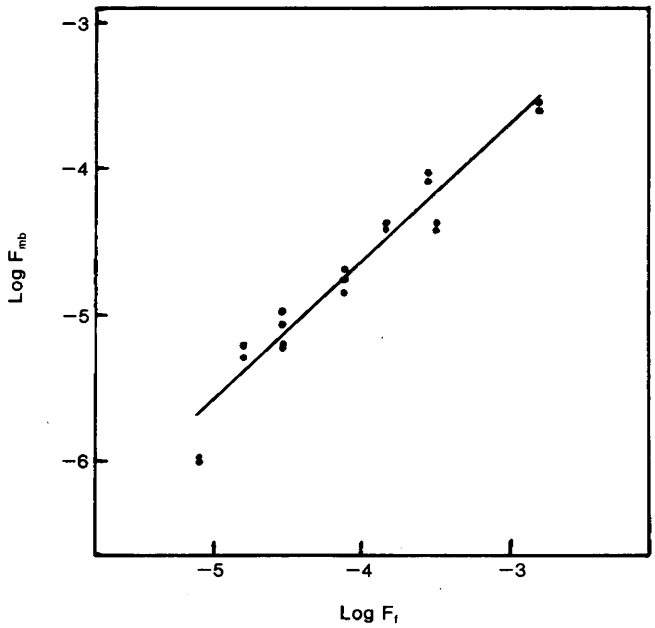


Fig 2.—Concentration-dependent binding of fluorescein by equilibrium dialysis cell membrane, determined in absence of serum proteins. Logarithm$_{10}$ of membrane-bound fluorescein (log F_{mb}) is plotted as function of the logarithm$_{10}$ of free fluorescein concentration (log F_f) at equilibrium. Data were fitted to straight line by method of least squares.

Fig 3.—Binding of fluorescein by human serum proteins at 37 °C, measured by equilibrium dialysis. Logarithm$_{10}$ of protein-bound fluorescein concentration (corrected for fluorescein bound to the dialysis membrane), log F_b, is plotted as function of logarithm$_{10}$ of free fluorescein concentration, log F_f, at equilibrium. Experimental curve (solid line), measured with serum protein diluted 1:10 in Tyrode's solution, has been corrected for dilution (binding by undiluted serum proteins) in upper curve (broken line).

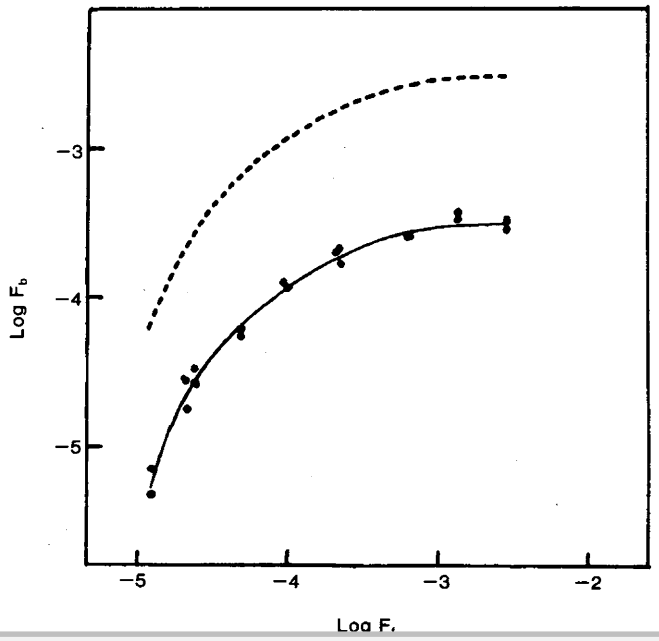
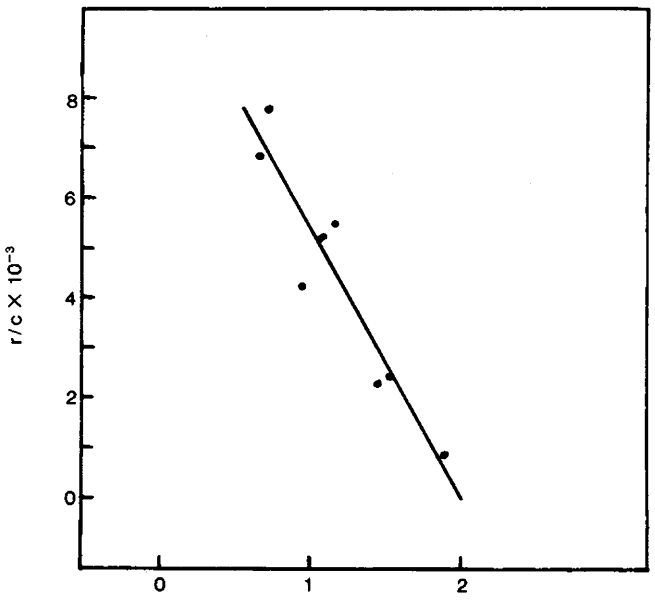


Fig 4.—Equilibrium dialysis data of Fig 3 presented in terms of Scatchard plot where r is amount of fluorescein bound by protein and c is free fluorescein concentration. Method of least squares was used to obtain best-fit straight line.



fluorescein (F_b) was obtained from the following relationship:

$$F_b = F_t - F_f - F_{mb}$$

where F_t is the total concentration of fluorescein, F_f is the concentration of free fluorescein at equilibrium, and F_{mb} is the fluorescein bound by the dialysis cell membrane.

RESULTS

The time required to obtain equilibrium of fluorescein diffusion across the dialysis membrane separating the two compartments of the equilibrium dialysis cell, determined in the group 1 experiments, was 50 hours (Fig 1). Therefore, all equilibrium dialysis experiments of fluorescein against serum proteins were continued for 50 hours or longer. The amount of fluorescein bound by the dialysis membrane, plotted against the free fluorescein concentration at equilibrium, determined in the group 2 experiments, is shown in Fig 2. The binding to the cellulose membrane was concentration dependent. This curve was used in calculating protein-bound fluorescein (F_b) from the experimental group 3 results, where the total fluorescein (F_t) and free fluorescein (F_f) concentrations at equilibrium were known.

In Fig 3, the logarithm of the protein-bound fluorescein concentration ($\log_{10} F_b$), corrected for the dye bound to the artificial membrane, is plotted against that of the free fluorescein concentration. The flatness of the binding curve at the highest free fluorescein concentrations indicated that protein fluorescein-binding site saturation had been obtained (Fig 3). The experimental data were determined using tenfold-diluted human serum proteins. To approximate the in vivo situation, the second (upper) curve of Fig 3 shows the data replotted for binding to undiluted serum proteins. This curve is simply obtained because the free fluorescein concentration (F_f) determines only the fraction of available binding sites occupied by fluorescein; the amount of fluorescein bound at a given free fluorescein concentration is a simple linear function of the concentration of available binding sites (eg, proteins) (see below, Fig 4).

The maximal and minimal fractions of the total fluorescein bound by undiluted serum proteins at 37 °C throughout the range of free fluorescein concentrations examined are given in the Table. The molar concentration of fluorescein-binding sites in undiluted human serum, determined from Fig 3, was 3.28×10^{-3}M.

Percentage of Total Fluorescein Bound by Undiluted Serum Proteins at Lowest and Highest Free Fluorescein Concentrations Measured

Fluorescein, M			
Total (F_t)	Free (F_f)	Bound (F_b)	Ratio $F_b/F_t \times 100$, %
6.9×10^{-5}	1.2×10^{-5}	5.7×10^{-5}	83
6.2×10^{-3}	2.9×10^{-3}	3.3×10^{-3}	53

Binding data of Fig 3 were replotted in Fig 4 in the form of a Scatchard plot,[11,12,14] r/c vs r, where r is the amount of protein-bound fluorescein and c is the concentration of free fluorescein. The maximum value for r (n) was arbitrarily chosen as 2, so that the association constant (K_0) is given by the reciprocal of the free-dye concentration at r = 1 (half of the binding sites of the proteins were occupied by fluorescein), as obtained from the relationship $K_0 = r(n - r)^{-1} c^{-1}$.[10,14] The average intrinsic association constant (K_0) of human serum proteins in a physiologic solvent at 37 °C for fluorescein was $0.54 \times 10^4 M^{-1}$.

COMMENT

The present results of equilibrium dialysis demonstrate that in a physiologic solvent at 37 °C, substantial binding of fluorescein to human serum proteins occurs throughout a wide range of fluorescein concentrations, even though the association is of low affinity. The appropriateness of equilibrium dialysis for evaluating fluorescein binding under physiologic conditions has been questioned by Ianacone et al[7] because of the long time required to obtain equilibrium. This misunderstanding has occurred because the time required for free ligand to reach equilibrium across the artificial dialysis membrane (eg, 50 hours) has not been differentiated from the forward rate constant for fluorescein-protein association, obtained when fluorescein is added directly to a protein solution, which is limited only by the rate of diffusion of fluorescein in free solution.[8-12] The artificial semipermeable dialysis membrane is only used so that an unambiguous measure of the free ligand concentration may be obtained. The technique is entirely appropriate for measurements of equilibrium thermodynamic parameters of association of rapidly interacting systems.[8-14]

Gel filtration and polyacrylamide gel electrophoresis,[7] however, may not be appropriate techniques to measure fluorescein-protein associations of low affinity. In both techniques, fluorescein-protein complexes are continuously being separated from free fluorescein because of their differential migration rates. This factor is particularly true in gel filtration, where fluorescein-protein complexes, because of their larger size, are continuously being transferred into solvent that contains no free fluorescein. This process is equivalent to dialyzing the fluorescein-protein complexes against an infinite volume of solvent and is an effective way to dissociate ligand-protein complexes of low affinity. These techniques will not measure the extent of binding of fluorescein to blood proteins that occurs in the presence of high concentrations of free fluorescein.

The concentration of fluorescein-binding sites in serum was determined to be 3.28×10^{-3}M. The normal concentration of albumin in human serum is 0.65×10^{-3}M.[15] Isolated bovine serum albumin has been reported to have three binding sites per molecule.[5] The ratio for bound fluorescein to human serum albumin of 5 (3.28:0.65) indicates either that human serum albumin has five fluorescein-binding sites per molecule or, more likely, that other serum proteins (eg, low-affinity IgG immunoglobulins) also bind fluorescein under physiologic conditions. A knowledge of the fluorescein binding by blood proteins of substantially different size and diffusion rates would be of importance for a complete picture in quantitative studies of blood-ocular permeability changes.

A knowledge of the extent of fluorescein binding to proteins at different free fluorescein concentrations also is necessary when blood fluorescein levels are measured fluorometrically, since these data must be corrected for the fluorescence quenching of the bound fluorescein if a true measure of total fluorescein content is to be obtained by this method.

The finding that a substantial fraction of the total injected fluorescein is bound at low affinity by human blood proteins in a physiologic solvent at 37 °C is consistent with clinical observations. The laminar flow seen in reti-

nal vessels[2,16] in all likelihood reflects fluorescein bound to proteins, as the protein-bound fluorescein diffusion rate will be much slower than that of free fluorescein. The rapid changes in the volumes of distribution of fluorescein and ^{131}I-labeled albumin, observed when both were injected simultaneously,[16] would reflect the low association constant of fluorescein binding.

A complete understanding of quantitative fluorescein angiography of the retina and vitreous fluorometry will require a detailed knowledge of the contribution of fluorescein binding by blood constituents during the changing concentration ratios occurring intravascularly and extravascularly after the initial injection of the bolus of fluorescein. The present experimental results may be used to calculate the contribution of fluorescein binding by human serum proteins in such studies.

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