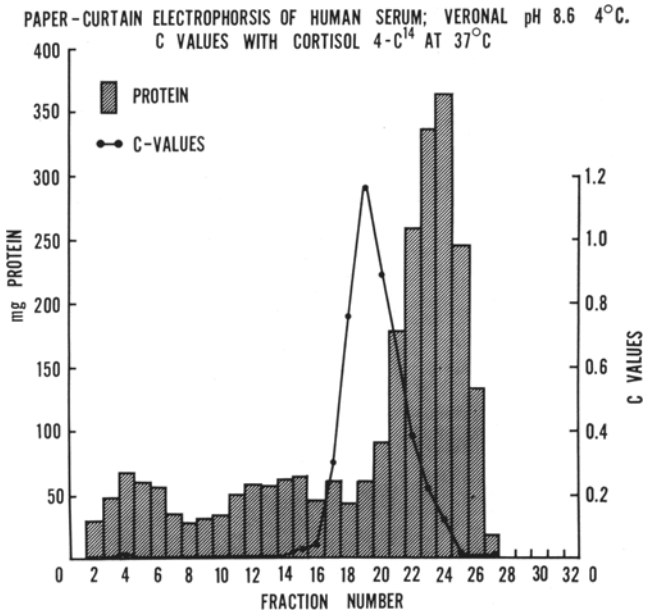

FIG. 31.

pH 8.6, the fractions 24, 25 and 26, obtained from the front shoulder of the albumin peak (Fig. 30) and containing the highest transcortin activity, migrated as α-globulin; fraction No. 24 had a small contamination of albumin, fractions No. 25 and 26 were free of albumin. This result restored the accordance with Figures 22 and 23. Paper-electrophoretic analysis of these fractions and whole rat serum at pH 7.8, 7.0, 5.5 and 4.0 revealed that a reversal of the relative electrophoretic mobility of the transcortin-containing α-globulin fraction and rat serum albumin occurs at about pH 7. A similar electrophoretic behavior has been observed for the α_1-acid glycoprotein which becomes a prealbumin at pH values of approx 7 and lower (29). In contrast, the transcortin-containing fraction isolated by continuous flow paper electrophoresis of human serum (Fraction 19 of Fig. 31) shows even at pH 4 an electrophoretic mobility slower than that of human albumin. The abnormal electrophoretic behavior of the transcortin-containing rat serum fraction on the paper curtain (Fig. 30) is interpreted as caused by a decrease of pH on the paper due to the low buffering capacity of the veronal buffer used (μ 0.02).

Although these results will have to be verified with the pure corticosteroid-binding globulins, they suggest characteristic differences in the physicochemical and biological properties of the transcortin systems of different species.

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Lipid-Protein Interactions[1]

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Abstract

Interaction of human serum albumin with phosphatidylserine was studied by turbidimetric methods. It was found that human serum albumin will bind phosphatidylserine. Analysis by the law of mass action for multiple equilibria has led to the conclusion that human serum albumin possesses a heterogeneity of binding sites for phosphatidylserine. The max number of sites and the respective affinity constants were calculated to be:

$K_1 = 2.0 \times 10^5$, $n_1 = 2$; $K_2 = 1.3 \times 10^3$, $n_2 = 30$

[1] Submitted in part as a thesis for the Ph.D. degree, University of Louisville. Carried out under the graduate training program of the U. S. Army Medical Research Laboratory.

Introduction

IN PRACTICALLY EVERY cell and tissue, complexes of protein with lipid may be found. Numerous studies have been carried out on protein-lipid interactions, including protein-phospholipid, protein-fatty acid, protein-steroid and protein detergent systems. In view of the occurrence of phospholipids in nearly all cells and the overwhelming evidence associating them with important biological phenomena such as the transport of various molecules across membranes (1–3), electron transport (4–7) and blood coagulation (8–14), the protein-phospholipid interactions would appear to be most interesting. However, progress has been slow in the study of the physiocohemical nature of the as-

sociation of phospholipid with protein because it has been extremely difficult to obtain pure samples. More detailed studies have been carried out using fatty acids, steroids and more particularly synthetic detergents produced by the attachment of a highly polar end group to long chain paraffins.

Protein—Fatty Acid Complexes. It has long been known that serum albumin interacts with fatty acid anions. As early as 1926, du Nouy (15) observed that the surface tension lowering effect, characteristic of fatty acid anions, was lost when soap solutions were added to serum, and surmised that there was interaction between the soap molecules and the serum proteins. In 1941, Kendall (16) found that crystalline human albumin was always associated with a small amt of free fatty acid that could not be removed by repeated crystallization. Teresi and Luck (17) obtained the first quantitative analysis of the interaction of several short-chain fatty acid anions with crystalline bovine serum albumin by the method of equilibrium dialysis. Unfortunately the fatty acid content of the albumin used in these experiments was not known. The fatty acids ordinarily associated with serum albumin would cause an error in the determination of the number of binding sites and of the strength of the interaction. More recently, Goodman (18), using the method of partition analysis, studied the interactions of long chain fatty acids with serum albumin, the fatty acid content of which was reduced to 0.1 moles/mole of albumin by extraction with 5% glacial acetic acid in isooctane. Treatment of his data by methods developed by Scatchard (19–21) allowed determination of the number of binding sites on each albumin molecule, and the apparent association constant for each class of sites. He found that each albumin molecule bears two sites with very high affinity for fatty acids, five with lesser affinity and approx 20 with very much less ability to bind fatty acids.

The mechanism involved in the binding of fatty acids to albumin is as yet unknown. That the nonpolar portions of the fatty acid contribute to its interaction with albumin seems well documented. Thus, Teresi and Luck (17) found that the binding increased with chain length for the fatty acids, acetate through caprylate. Goodman (18) also found the apparent association constant for the homologous saturated fatty acids laurate through stearate, increasing with increasing chain length. This effect of the nonpolar portion of an anion usually has been ascribed to the summation of a number of short range non-specific van der Waals interactions between the nonpolar portions of the binding ion and the non-polar side chains of the albumin molecule. Though it has not been rigorously proven, it is presumed that the fatty acids are bound to albumin as anions. The relatively strong interaction would be difficult to explain on any other basis than an association of the carboxylate anion and some positively charged group on the surface of the albumin molecule. This is supported by findings of Ballou et al. (22) who have shown by electrophoretic studies that a combination of fatty acids with albumin causes an increase in the mobility of serum albumin, proving that the albumin fatty acid complex is more negative than is albumin alone. As yet, however, no data are available from which the fatty acid binding sites on albumin can be deduced.

Protein-Phospholipid Complexes. Studies of protein-phospholipid interactions have not been as complete or as quantitative as those we have mentioned thus far for fatty acids. Many attempts have been made to form complexes between isolated phospholipid fractions and various proteins. Chargaff (23) demonstrated that cephalin preparations from brain formed water insoluble salts with the highly basic protamine, salmine. That the precipitation of cephalin was not a salting out effect was shown by the fact that the isolated precipitates could not be reemulsified in water. In a similar study, Chargaff and Ziff (24) investigated the interaction of lecithin and cephalin with other proteins such as histones from calf thymus and globin from cattle hemoglobin. They demonstrated that histone formed insoluble compounds with cephalin between pH 2 and 7, whereas with globin appreciable formation of insoluble cephalin compounds was observed only below pH 4. In contrast, lecithin formed no compounds with globin, but did so with histone between pH 7 and 8. Mayer and Terroine (25) found that a lecithin emulsion precipitated albumin in acid solution. The precipitate was shown to contain albumin and lecithin, but quantitative studies were not made. A serum albumin-lecithin complex was also obtained by Haefer (26) when lecithin dissolved in alcohol was added to a solution of serum albumin. Fujii (27) showed that lecithin in the presence of albumin was no longer water-dispersible, but, rather, the two substances formed an ether-soluble product. Spiegel (28,29) also studied the interaction of lecithin and cephalin with serum albumin. He found that, unlike lecithin, cephalin is not flocculated by serum albumin. He also demonstrated that cephalin is able to prevent heat coagulation of serum albumin. Maurer and Muller (30) found that the addition of albumin to rat and rabbit sera profoundly altered the distribution of phospholipid in that the phospholipids initially associated with the globulins appeared to form a complex with added albumin. Florsheim et al. (31) also demonstrated by electrophoretic techniques that albumin is capable of binding lipids from deproteinized human lipoprotein.

Most studies thus far have been poorly defined and few physicochemical measurements have been made; however, Inoue (32,33) found that electrophoretic mobilities of soluble complexes of purified lecithin and crystalline ovalbumin were intermediate between that of lecithin and ovalbumin. The presence of lecithin was also found to prevent the heat coagulation of ovalbumin. In addition, Eley and Hedge (34) studied lipid protein interactions by means of interfacial tension measurements at air-water interface. The systems studied by Eley and Hedge consisted of bovine plasma albumin and synthetic dipalmitoylphosphatidylethanolamine and distearylphosphatidylcholine. They observed that a monomolecular layer of albumin is able to associate with 330 molecules of phosphatidylethanolamine, and concluded that each molecule of phosphatidylethanolamine interacts with two peptide residues through their amino and phosphate groups either by hydrogen bonding or ion-dipole association. However, the molecular ratio of lecithin to albumin was only half that obtained for phosphatidylethanolamine. Since this agrees well with the number of ionized carboxyl groups available in the albumin molecule, they suggested that lecithin interacts by ionic association of the positively charged groups of lecithin and the negatively charged groups of the protein.

Phosphatidylserine-Albumin Interactions

Quantitative as well as qualitative information regarding the physicochemical nature of the association

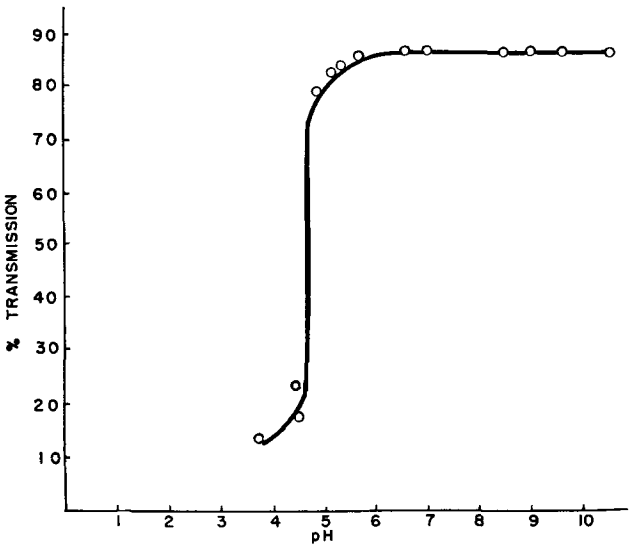

FIG. 1. Turbidity of a mixture of phosphatidylserine and albumin measured at diffeernt pH values.

of phospholipid with protein can only be obtained if all components in the system are pure. The purification of phosphatidylserine from beef brain either by chromatographic methods (35) or by countercurrent distribution (36) has made it feasible to study the nature of the interactions between this highly purified phospholipid and crystalline serum albumin.

Experimental Procedure

Materials. Purified phosphatidylserine from beef brain was prepared by countercurrent distribution as previously described by Therriault et al. (12). Lipid was removed from crystalline human serum albumin by extraction with chloroform-methanol followed by treatment with cysteine according to the method of Terriault and Taylor (37).

Turbidimetric Determination of Binding. Turbidity measurements were made with a Brice-Phoenix light scattering photometer at 546 mμ in a standard turbidity cell 30 x 30 x 60 mm. The light transmitted at 0° angle was determined and the turbidity calculated as 1 minus transmission. A series of emulsions were prepared at desired concn by appropriately diluting the stock phosphatidylserine emulsion with phosphate buffer pH 7.3 ionic strength 0.1. In order to determine the turbidity of phosphatidylserine in the presence of albumin, various amt of the stock phosphatidylserine were added to a series of cells containing a known amt of lipid free albumin and adjusting the mixture to a constant volume with phosphate buffer. The concn of the albumin in stock solutions was determined by absorption at 280 mμ. The value of $E^{1cm}_{1\%}$ was found to be 5.45. For calculations of M concn, the mole wt of serum albumin was taken as 65,000.

Paper Chromatography. Chromatographic analysis was carried out at 0C on silicic acid impregnated paper with a solvent mixture of diisobutyl ketone: acetic acid: water in the ratio 40:20:3 by volume, according to the method of Marinetti et al. (38). The chromatograms were dipped into a 0.001% aqueous solution of rhodamine 6G and examined under UV light. The original purified phosphatidylserine sample was used as a standard for comparison of R_f values. To detect the free amino group of serine, papers were sprayed with a 0.3% solution of ninhydrin in *n*-butanol saturated with water and containing 10% lutidine.

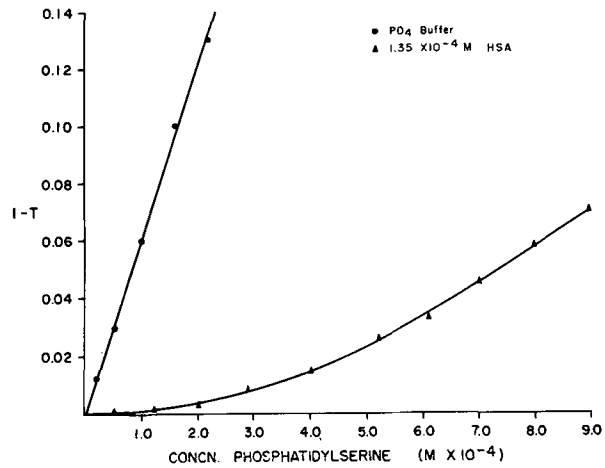

FIG. 2. Effect of albumin on turbidity of phosphatidylserine emulsions. Measurements made at pH 7.3—HSA = human serum albumin.

Phosphorus Determination. The solvent was evaporated in a hot water bath using a glass bead in each test tube to prevent bumping, and the phosphorus content was determined as described elsewhere (12).

Results

When phosphatidylserine, at pH 3.2, was added to an unbuffered 1.0% solution of albumin in 0.2 M NaCl at pH 4.6, the pH immediately came to 3.8 and a precipitate formed. The precipitate could be caused to redissolve merely by raising the pH. This phenomenon of reprecipitation and resolubilization could be repeated indefinitely by adjusting the pH with either dilute alkali or acid. The pH at which precipitation takes place is shown in Figure 1. The percentage transmission of a mixture of phosphatidylserine and albumin was measured after adding small increments of 0.01 N NaOH and determining the pH. It is seen that precipitation take place below the isoelectric point of albumin (pH 4.6) whereas the complex is soluble above that pH. In the binding studies, therefore, all measurements were made at pH 7.3. The method employed in the present study of the binding of phosphatidylserine by human serum albumin is based on the observation that a suspension of phosphatidylserine forms a clear solution on addition of serum albumin. The line represented by the solid triangles in Figure 2 is an illustration of the turbidity determinations obtained with increasing concn of phosphatidylserine in a 1.35 x 10^{-4} M serum albumin solution. It is seen that the turbidity of a given concn of phosphatidylserine is considerably decreased in the presence of albumin. This decrease in turbidity is attributed to the interaction of insoluble phosphatidylserine with serum albumin to form a soluble complex.

Assuming that the particle size of the insoluble phosphatidylserine is the same in phosphate buffer as it is in the presence of albumin, and that a decrease in turbidity is a result of interaction with albumin only, the amt of phosphatidylserine bound to a given amt of albumin can be determined from Figure 2. The amt of free phosphatidylserine also can be determined by difference between the amt bound and the total amt present. The mole ratio of phosphatidylserine to albumin was calculated and also the value of r/I which is the ratio of the moles phosphatidylserine bound/mole protein to the moles of unbound (free) phosphatidylserine. These values show in Table I.

TABLE I

Binding of Phosphatidylserine by Serum Albumin

Concn M x 10^{-4}			Avg mole P.S. per mole protein r	$\frac{r}{I}$ x 10^{-4}
Total P.S.	I Free P.S.	Bound P.S.		
0.275	0.005	0.270	0.20	40.0
0.690	0.015	0.675	0.50	33.3
1.310	0.04	1.35	1.00	25.0
2.000	0.07	1.93	1.43	20.4
3.000	0.14	2.86	2.12	15.1
4.000	0.25	3.75	2.78	11.1
5.000	0.39	4.61	3.42	8.8
6.000	0.56	5.44	4.03	7.2
7.000	0.74	6.26	4.64	6.3
8.000	0.93	7.07	5.24	5.6
9.000	1.15	7.85	5.82	5.1

Concn of human serum albumin 1.35 x 10^{-4} M.

In dealing with the attraction of proteins for small molecules and ions, it is recognized that the protein is capable of combining with more than one of the interacting ions or molecules. Therefore, the law of mass action, as applied to numerous equilibria which may be involved, becomes quite complex in form. Klotz has derived the relationships for the application of the law of mass action to the binding by proteins (39). By making certain simplifying assumptions that the bound molecule exerts no electrostatic influence on the succeeding binding and that each site on the protein has the same affinity for the molecules, the following equation was derived:

$$r = nK(I)/[1 + K(I)] \quad [1]$$

The value of n represents the average max number of sites/molecule, K is the association constant, r is the number of molecules bound/molecule protein and (I) is the concn of unbound (free) molecules at equilibrium.

In general, of course, the situation is somewhat more complex than is represented here. Most macromolecules, such as proteins, are likely to contain, not one single set of binding sites, but several such sets. Scatchard (20), in dealing with this situation, has derived the following equation for the binding of an ion to several classes of sites on a protein molecule

$$r = \sum_i ri = \sum_i ni\,Ki(I)/[1 + Ki(I)] \quad [2]$$

in which K is the apparent association constant for each of the n sites in class i under any particular set of conditions. On the asumption that the binding may be decribed by two different values of K, the operation may written as follows:

$$r/I = n_1K_1/[1 + K_1(I)] + n_2K_2/[1 + K_2(I)] \quad [3]$$

In a situation where there is but one class of binding sites, a plot of r/I as ordinate against r as abscissa gives a straight line. The intercept of the abscissa gives n; the intercept on the ordinate gives Kn from which K can be derived. The analysis is useful in situations where there are several classes of binding sites as well, for here there will be a deviation from the straight line relationship.

The data obtained from Table I are plotted in Figure 3 in terms of equation 2. The linear relationship of the initial portion of this curve confirms that in this area the binding follows the simpler formulation. The max number of sites for this step in the binding curve is indicated to be 2 and the value of K found here is equal to 2.0 x 10^5. The broken line is obtained by substitution of these values into the previous equation and, therefore, represents the theoretical straight line for n = 2, K = 2.0 x 10^5. At higher values of r, a linear extrapolation of the curve will permit an estimate of n_2. This extrapolation

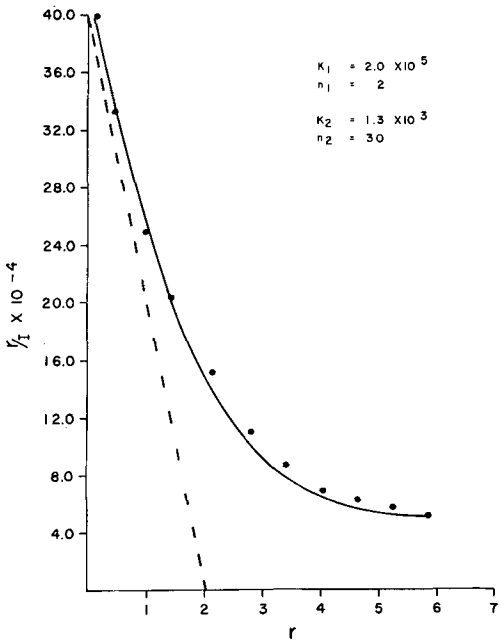

FIG. 3. Plot of r/I vs. r, according to Scatchard. Closed circles are the experimentally determined points. Broken line represents the theoretical curve for K = 2.0 x 10^5, n = 2. Solid line represents the theoretical curve for two constants,

$K_1 = 2.0 \times 10^5$, $n_1 = 2$ and $K_2 = 1.3 \times 10^3$, $n_2 = 30$.

yields $n_2 = 30$. The value of K_2 is obtained from equation 3 and is equal to 1.3 x 10^3. The theoretical curve obtained with these affinity constants, K_1 and K_2 is represented by the solid line. The closed circles are the experimentally determined points. It is seen that the curve calculated on the basis of the two association constants fits the experimental points satisfactorily. Though the values of k, and n, can be determined with some degree of reliability (±5%), the values for n_2 and k_2 should be considered only as an estimate. The inherent error in estimating the n_2 and k_2 values is not due to the turbidimetric method, which is reproducible to ±5%, but rather to the long extrapolation which is necessary to arrive at these values.

To test the assumption that the decrease in turbidity is due to solubilization of the insoluble phosphatidylserine as a result of its interaction with serum albumin, mixtures of phosphatidylserine and albumin were centrifuged in order to sediment the undissolved particles. The clear supernatant albumin solution was then lyophilized and the dry powder extracted with a mixture of chloroform-methanol 2:1 by volume. A portion of the extract was evaporated to dryness under a stream of nitrogen, taken up in a small quantity of chloroform-methanol and chromatographed on silicic acid impregnated paper. A single spot could be detected on the chromatogram corresponding in R_f value to the original phosphatidylserine sample and gave a positive reaction with ninhydrin solution. Phosphorus determinations which were run on the remainder of the chloroform-methanol extract revealed that the amt of phosphatidylserine, calculated on the basis of mole wt 800, extracted from the albumin solution with chloroform-methanol correspond to within 20% of the amount which was determined from turbidimetric measurements.

Estrone-Albumin Binding. Further confirmation of the applicability of this method has been obtained on studies of estrone binding with bovine serum albumin. The binding of estrone was studied by Sandberg et al.

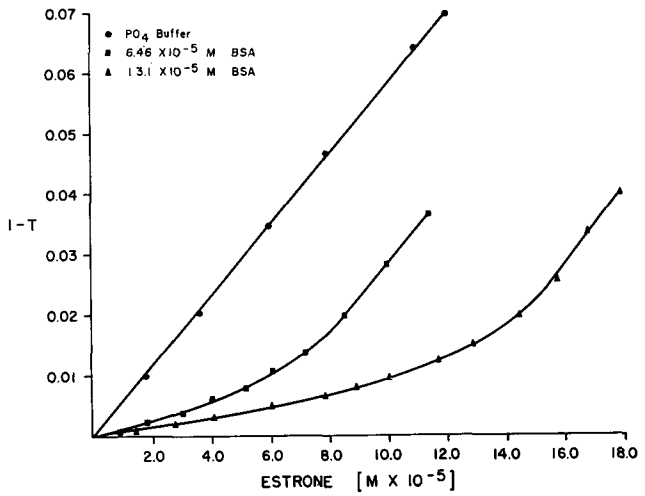

FIG. 4. Effect of albumin at two different concn on the turbidity of estrone suspensions.

(40) by means of equilibrium dialysis. Due to its extremely low solubility, they were limited to very low concn of estrone and consequently small values of r. The association constant determined from these results was 6.0 x 10^4 with n equal to 1.0. The free energy of association was $\Delta F = -6.1$.

Figure 4 shows the turbidity measurements of various concentrations of estrone in phosphate buffer pH 7.3 compared to the turbidity in two different concentrations of bovine serum albumin. It is interesting that the point of intersection of the two slopes in either curve shows a 1:1 relationship between albumin and estrone indicating one binding site on the albumin molecule. Analysis of these results by means of the Scatchard plot (Fig. 5) permits a determination of the association constant and the number of sites. It is seen that these are in close agreement with those reported by Sandberg et al. (40).

Discussion

The calculated affinity constants suggest that phosphatidylserine binding sites on human serum albumin may be divided into two distinct groups. The first group would consist of 2 sites/ protein molecule with a relatively high affinity. The second group consists of ca. 30 sites having about 150 times lower affinity. It would be carrying the interpretation beyond the limits of the experimental methods used to infer that the sites in each group have identical constants. Rather, it may be more judicious to regard K_1 and K_2 as average values around which the individual values in each group are distributed. The calculation of two separate classes of binding sites does not exclude the possibility that there may still exist more binding sites. If, however, there are more sites in the protein capable of binding phosphatidylserine, then it may be expected that their binding constants will be substantially less than the smaller of the two constants.

It should be mentioned again that the results of Eley and Hedge (34) indicated that one molecule of bovine serum albumin spread in a monomolecular layer interacted with 330 molecules cephalin. They suggested that the complexes could occur through

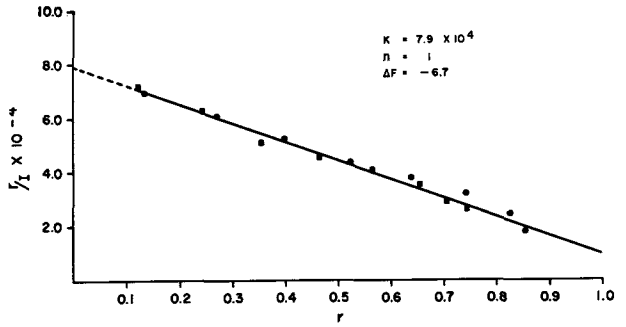

FIG. 5. Plot of r/I vs. r, according to Scatchard. Squares represent values calculated from determinations at an albumin concn of 6.46 x 10^{-5} M. Circles are from an albumin concn of 13.1 x 10^{-5} M.

strong hydrogen bonds or by ion-dipole association between the two charged groups of cephalin and the peptide bond dipoles. The large number of binding sites found by these investigators, compared to that found in the present study, may be due to the fact that a monomolecular layer of albumin is in an expanded state exposing a greater number of sites for interaction, whereas in the native globular state, most of the sites are not available and cannot interact.

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