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Peptide–Protein Interaction as Studied by Gel Filtration*

Gordon F. Fairclough, Jr.,† and Joseph S. Fruton

ABSTRACT: The gel-filtration method of Hummel and Dreyer has been used for the determination of the binding constants in the interaction of bovine serum albumin with L-tryptophan or tryptophan derivatives, including peptides. This method, as modified in the present work, appears to be at least as precise as dialysis equilibrium and, when automated, offers many advantages in speed, convenience, and flexibility. Evidence is offered for the attainment of equilibrium conditions in the operation of the gel-filtration method, and for its utility in the quantitative measurement of the competition of two small molecules for a protein binding site. The results of earlier dialysis–equilibrium studies on the binding of L-tryptophan and of acetyl-L-tryptophan by serum albumin, by McMenamy and his associates, have been largely confirmed in the present work. Data are also given for the primary association constants in the binding, to bovine serum albumin, of D-tryptophan, acetyl-L-tryptophanamide, acetyl-D-tryptophanamide, and several tryptophan-containing peptides. The available evidence suggests that serum albumin has a single strong binding site for L-tryptophan, and that the primary binding of tryptophan derivatives occurs at this site.

The specific interaction of peptides with proteins is a phenomenon of considerable biochemical importance. One of the most striking demonstrations of such interaction is the combination of the S-protein and S-peptide of ribonuclease to regenerate enzymic activity (Richards and Vithayathil, 1959). This discovery, together with the work of Smyth *et al.* (1963) in establishing a revised amino acid sequence for the S-peptide, has been followed by the work of Hofmann's group (Finn and Hofmann, 1965), who have examined the ability of various synthetic peptides to replace the intact S-peptide in the regeneration of ribonuclease activity. It has been suggested (Hofmann, 1962) that some peptide hormones may act *in vivo* by virtue of their ability to interact specifically with "receptor" proteins and, by analogy to the case of ribonuclease-S, to cause the generation of enzymic activity. Evidence has been adduced for the specific binding of the peptide hormones oxytocin and vasopressin by a protein fraction (neurophysin) of beef posterior pituitary (for a recent report, see Ginsburg and Ireland, 1964).

From a more general point of view, the study of the specific interaction of peptides with proteins has significance for several central problems of protein chemistry. Among these are (1) the contribution of specific interaction between parts of a long peptide chain so as to confer upon it a characteristic conformation (Schellman and Schellman, 1964); (2) the specific interaction between protein (and peptide) antigens and the antibody γ-globulins elicited upon their administration to suitable animals; (3) the interaction between enzymes that act at peptide bonds and their substrates, leading to specificity of enzymic catalysis. For all these problems, more systematic quantitative studies are needed, in which the binding of synthetic peptides of known structure to well-defined proteins has been examined.

In the specific interaction of peptides with proteins, at least four types of bonding may be envisaged: (1) electrostatic interaction between oppositely charged ions; (2) interaction between apolar side-chain groups; (3) hydrogen bonding either involving side-chain groups such as those of tyrosine and glutamic acid, or involving CO–NH groups; (4) interaction between CO–NH groups and aromatic structures (Robinson and Jencks, 1965). With multifunctional peptides, it may be expected that cooperative bonding through several groups of the peptide will occur, provided the stereochemical requirements for such multifunctional binding are met by the

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protein under study. It may also be expected that, in consequence of such multifunctional binding, the peptide chain of the protein will undergo conformational changes.

Although the above considerations have been discussed previously, little quantitative work appears to have been done to study the effect of changes in peptide structure on peptide–protein interaction. Furthermore, most of the studies on the binding of small organic molecules by proteins have dealt with ions, and relatively less information is available on the binding of uncharged molecules.

A major requirement for the systematic study of the binding of peptides by proteins is the availability of reliable quantitative methods for the determination of the association constants of the peptide–protein complexes formed. In quantitative studies on the binding of ions by proteins, the dialysis–equilibrium method has been used most widely (for a recent discussion of the advantages and limitations of this method, see Steinhardt and Beychok, 1964). A valuable alternative method was described by Hummel and Dreyer (1962), who used gel filtration for the study of the binding of cytidine 2′-phosphate by ribonuclease. A similar procedure was employed by Pfleiderer (1964) for the study of the binding of reduced diphosphopyridine nucleotide (DPNH) by lactic dehydrogenase.[1]

The method of Hummel and Dreyer (1962) involves the equilibration of a Sephadex column with the substance of low molecular weight, and the protein (dissolved in the solution used to equilibrate the column) is applied. Elution of the column with the same solution as that used for equilibration leads to the emergence of the protein at the excluded volume of the column, followed in the elution diagram by a trough whose area gives a measure of the amount of small molecule bound by the protein. In the present communication, we report the application of the gel-filtration method to the study of the binding of L-tryptophan and its derivatives by bovine serum albumin (BSA). The details of the procedure, as modified for our studies, are given in the Experimental Section.

This investigation was undertaken to examine the potentialities of the gel-filtration method for the quantitative study of peptide–protein interaction. The tryptophan–serum albumin system was selected because of the extensive previous work, largely by McMenamy and Oncley (1958), McMenamy (1963, 1964), and McMenamy and Seder (1963), on this system. By means of the dialysis-equilibrium method, they showed that BSA and human serum albumin (HSA) strongly bind 1 mole of L-tryptophan per mole of protein, and they also examined the binding of various tryptophan derivatives (acetyl-L-tryptophan, L-tryptophan methyl ester, D-tryptophan methyl ester, indole propionate, tryptamine, and skatole) by serum albumin. As an initial approach to the use of the gel-filtration method to the quantitative study of the binding of peptides by serum albumin and other proteins, we have examined the binding of L-tryptophan, D-tryptophan, acetyl-L-tryptophan, glycyl-L-tryptophan, glycyl-L-tryptophanamide, L-tryptophylglycyl-L-tryptophanamide, and glycyl-L-tryptophylglycyl-L-tryptophanamide, as well as uncharged compounds such as acetyl-L-tryptophanamide and glycyl-L-tryptophan diketopiperazine.

Experimental Section

Materials. Except where otherwise noted, the sample of BSA was a preparation from Pentex Co. (lot No. BX3). We are informed by the manufacturer that a stabilizer such as caprylate was added in the initial processing but was removed during the 4–5 subsequent crystallizations. Through the kindness of Dr. R. H. McMenamy, we received a sample of BSA (Armour, lot No. Y20907); he has informed us that the protein had been subjected to extensive dialysis against KCl solutions containing 1 mM EDTA and then deionized by the mixed-bed resin technique. The water content of the two protein preparations was determined (5.9 and 4.7%, respectively), and all data are expressed in terms of the dry weight of protein used. A molecular weight of 69,000 was assumed.

The following compounds were commercial preparations: L-tryptophan (Winthrop Laboratories), D-tryptophan (Mann Research Laboratories), glycyl-L-tryptophan (Hoffmann LaRoche Co.), acetyl-L-tryptophan, mp 185–186° (Mann Research Laboratories), acetyl-L-tryptophanamide, mp 192–193° (Mann Research Laboratories). They appeared to be homogeneous, as judged from their chromatographic behavior. Acetyl-D-tryptophanamide was prepared from D-tryptophan according to the method described for the L compound (Huang and Niemann, 1951); it melted at 193–194°, in agreement with the value reported for the enantiomer. Glycyl-L-tryptophan diketopiperazine was prepared by dissolving 0.41 g of glycyl-L-tryptophan methyl ester *p*-toluenesulfonate (Theodoropoulos and Fruton, 1962) in 15 ml of methanol previously saturated with NH_3 at 0°. After 2 days at room temperature, the reaction mixture was concentrated under reduced pressure, and the residual product was crystallized from water. After recrystallization from water, it melted at 287–288°; yield 0.1 g (48%). (*Anal.* Calcd for $C_{13}H_{13}N_3O_2$: N, 17.3. Found: N, 17.0.)

Glycyl-L-tryptophanamide, L-tryptophylglycyl-L-tryptophanamide, and glycyl-L-tryptophylglycyl-L-tryptophanamide were available as *p*-toluenesulfonates, and had been prepared as described by Theodoropoulos and Fruton (1962). Before using these compounds in binding studies, it was necessary to remove the *p*-toluenesulfonate ion, which may be expected to be bound strongly by BSA (Klotz *et al.*, 1948). To effect this removal, solutions of the salts were passed through Sephadex G-25 columns (0.9 × 34 cm); the *p*-toluenesulfonate emerges first, and the tryptophan peptides are retarded (Ruttenberg *et al.*, 1965). With the tri- and tetrapeptide amides the separation was complete in one operation; in the case of glycyl-L-tryptophanamide,

[1] Abbreviations: bovine serum albumin, BSA; human serum albumin, HSA; diphosphopyridine nucleotide reduced, DPNH.

the dipeptide amide fraction was passed through Sephadex G-25 once more to remove the last traces of *p*-toluenesulfonate.

Determination of the molar extinction coefficients at 280 mμ of L-tryptophan and its derivatives at pH 9 (cell path lengths, 0.05–1.0 cm) gave the following values: L-tryptophan and D-tryptophan, 5560; acetyl-L-tryptophan, 5180; acetyl-L-tryptophanamide and acetyl-D-tryptophanamide, 5560; glycyl-L-tryptophan diketopiperazine, 5450; glycyl-L-tryptophan, 5030. For glycyl-L-tryptophanamide, L-tryptophylglycyl-L-tryptophanamide, and glycyl-L-tryptophylglycyl-L-tryptophanamide, a molar extinction coefficient (per tryptophan residue) of 5560 was assumed. These values were used for the spectrophotometric determination of the concentration of the above compounds in the operation of the gel-filtration method. Where comparison is possible, there is satisfactory agreement with the data in the literature (Beaven and Holiday, 1952). In the concentration ranges used for the various compounds, Beer's law was obeyed; this was taken to indicate that under the conditions of these studies, the extent of aggregation of the small molecules was negligible. For the gel-filtration studies near pH 9, 0.125 M ethanolamine–HCl buffers (0.1 M in chloride) were used. The ethanolamine was distilled before use. For the experiments at pH 6, 0.1 M KCl solutions were used. Glass-distilled water was boiled before use to make up all solutions employed in this work.

Sephadex Columns. Units consisting of four Sephadex G-25 columns (0.5 × 115 cm) within a single constant-temperature jacket were used to obtain binding data. To one end of a standard 48-in. length of glass tubing (i.d., 0.5 cm), was fused the socket portion of a 12/5 ground-glass ball-and-socket joint. The other end was tapered and fitted with an 8-in. length of Teflon tubing (i.d., 1.2 mm). Glass wool was packed gently into the tapered end and covered with a porous Teflon disk (4 mm thick) to form a base for the column of Sephadex G-25.

A 500-ml capacity aspirator bottle with 24/40 outer joints and fitted with an exit stopcock was used as the reservoir for each column. The outlet of the stopcock was extended 3 in. by fusing to it a length of 0.25-in. diameter glass tubing, and connected by a 3-in. length of 0.25-in. diameter Teflon tubing to the ball portion of a 12/2 ground-glass ball-and-socket joint, which mates with the 12/5 socket on the column. A constant-pressure head was maintained during a run; for the runs at alkaline pH the air was first passed through a sodalime trap before it bubbled into the reservoir.

The constant-temperature jackets were made from standard 48-in. length glass tubing (i.d., 1.5 in.) by attaching inlet and outlet ports 3 in. from the bottom and top ends of the jacket. Size No. 8 rubber stoppers, with holes to accommodate the four columns, were used to make water-tight seals at the ends. The four Sephadex columns are entirely within the jacket, the tops of the columns being at the level of the outlet port. From a central-fifth hole in the top stopper an immersion thermometer (0–50°, calibrated in tenths) was suspended in the middle of the jacket, its bulb being 17 in. from the top. Water was circulated through the system at a preset temperature by means of a constant-temperature circulating bath (Precision Scientific Co.). For the runs at the temperatures reported, the bath temperature and the jacket temperature differed by less than 0.05°.

For the preparation of four columns, 40–45 g of dry Sephadex G-25 were suspended in 1 l. of 0.10 M NaCl in a 2 l. side-arm flask. To deaerate the Sephadex, the suspension was placed under reduced pressure (water aspirator) and stirred slowly with a bar magnet for 3–6 hr. The fine particles were decanted after a 10–15 min settling time. Approximately 250 ml of deaerated 0.10 M NaCl was used to resuspend the Sephadex and the settling and decanting was repeated. This was continued until the supernatant was clear (usually 10 such washes were sufficient).

A funnel was connected to each of the four glass assemblies by a short piece of rubber tubing and the ball portion of a 12/5 ground-glass ball-and-socket joint. To form a column of liquid, free of trapped air bubbles, the column was filled through the exit tubing from an elevated reservoir of deaerated 0.10 M NaCl, and the liquid was allowed to flow up the glass tubing to the funnel. Then, with the exit tubing stoppered, the Sephadex slurry was divided in equal amounts between the four funnels and allowed to settle through the static liquid column. When the Sephadex was packed under the influence of gravity to a height of 5–10 cm, the stoppers were removed from the exit tubing, and the packing was allowed to continue under the added influence of liquid flow. When the packed columns of Sephadex reached the ball-and-socket joints, the funnel assemblies with excess Sephadex were removed, the columns were connected to a reservoir of 0.10 M NaCl, and 250 ml of solution was passed through each column to further settle the bed. Excess Sephadex was then removed from each column to give a matched set of four columns with a bed height of approximately 115 cm. To stabilize the top of the Sephadex bed, a porous Teflon disk (4-mm thick) was placed on the top.

General Procedure for a Binding Run. The description will be for a single column run, although four runs may be done simultaneously at the same temperature. The column was equilibrated at the desired temperature with a known concentration of tryptophan (or its derivative) by passing through the column a volume of solution equivalent to 1.5–2 times the column-elution volume for that particular compound. A sample of BSA was weighed into a 5-ml beaker and dissolved in 1.0 ml of the solution used to equilibrate the column. After 30 min at room temperature, the protein solution was transferred to the top of the Sephadex column, and 10 min later, for adjustment to the temperature of the column, the protein solution was allowed to run into the column. The beaker was washed with 0.2 ml of solution, and the wash solution was also run into the column. The reservoir was reconnected to the column and elution with the solution used to equilibrate the column was allowed to proceed. The flow rate of the Sephadex

columns varied from 4–5 ml/hr at 15° to 9–11 ml/hr at 40°. By means of a fraction collector, the effluent solution was collected in serial fractions of slightly under 1 ml, the usual range being from 0.70 to 0.95 ml. Best results were obtained if evaporation was lessened by storing the tubes in a refrigerator shortly after their collection. The pH values of several fractions in the portion of the elution diagram containing the protein were determined at room temperature (usually 25°) within 30 min after their collection and before being stored in the cold. After the run was about half-over, the serial reading of the absorbance of each fraction at 280 mμ was begun using the Beckman DU spectrophotometer and cells of suitable path length. After all absorbance readings were made, the volume of each fraction in the region of the trough was determined by drawing the entire fraction into a 1-ml pipet calibrated to the tip and previously wetted with buffer.

Recently the method has been modified to provide automatic and continuous monitoring of the absorbance of the effluent solution at 280 mμ by means of a Gilford-2000 multiple sample absorbance recorder equipped for column chromatography with Model 208 auxiliary offset control, Model 215 automatic blank compensator, and Model 203 flow cell assembly with "standard" Kel F cells blocks (Gilford Part No. 1047). The clearance characteristics of these flow cells are well suited to the monitoring of elution samples having the relatively large amounts of protein in the small volume required by this method. To ensure the absence of air bubbles in the flow cells, the solutions to be used in binding runs were deaerated under reduced pressure (water aspirator) and the cell compartment was maintained at the temperature of the columns. It has been found most convenient to monitor the absorbance of the effluent profile relative to an air blank. Usually only a single column was monitored at a time, but multiple recording of runs has been done successfully. For each run the relationship between the column flow rate and the recorder-chart advance rate was determined by indicating on the chart the advancement made during the collection of 5.00 ml of column effluent in a volumetric flask. This determination is routinely made during the elution of the trough; however, identical flow rates have been determined before and after the elution of the trough in given experiments.

With the Sephadex columns employed in these studies, the protein peak usually emerged at 8–9 ml of the elution pattern, and the minima of the troughs for the small molecules were: L-tryptophan and D-tryptophan (28 ml), acetyl-L-tryptophan (23 ml), acetyl-L-tryptophanamide (30 ml), glycyl-L-tryptophan diketopiperazine (35 ml), glycyl-L-tryptophan (24 ml), glycyl-L-tryptophanamide (32 ml), L-tryptophylglycyl-L-tryptophanamide (68 ml), glycyl-L-tryptophylglycyl-L-tryptophanamide (64 ml).

Calculations. The amount of tryptophan compound bound by the BSA sample during a given run was determined from the area of the trough in the elution diagram. For the binding runs in which individual serial fractions of the column effluent solution were collected, the following procedure was used. All observed absorbance values were corrected for any apparent absorbance due to the buffer alone and the use of unmatched cuvets. The average of the corrected absorbance values of fractions in two 5-ml portions of the elution diagram, one before the trough and the other after the trough, was taken as the base-line absorbance value. The trough is considered to have started when the absolute value of the difference between the absorbance of a given fraction and the base-line absorbance is greater than the average deviation from the mean for all the values used to determine the base-line value, and to have stopped when the difference is less than the average deviation from the mean The difference between the base-line absorbance and the absorbance of each fraction constituting the trough was determined and used in the equation

$$\text{apparent } \mu\text{moles bound} = \frac{\sum_i(\Delta A_i \times ml_i)}{\epsilon \times 10^{-3}}$$

where ΔA_i is the difference between the base-line absorbance value and that of fraction i of the trough; ml_i is the volume of fraction i; ϵ is the molar extinction coefficient of the tryptophan compound as determined with the particular cell used for the absorbance measurements of the column effluent. The molar concentration of small molecule with which the protein is in equilibrium on the column was determined by dividing the average base-line absorbance by the molar extinction coefficient for the small molecule; this concentration is designated c. The value of c determined in this manner was within 1% of that calculated from the absorbance of the solution used to equilibrate the column.

For binding runs in which the elution profiles were recorded automatically, the area of the trough (in square inches) was determined by graphical integration and used in the equation

$$\text{apparent } \mu\text{moles bound} = \frac{\text{area of trough}}{ab(\epsilon \times 10^{-3})}$$

where a is the pen excursion in inches for a change in absorbance of 1.00 and b is the chart advancement in inches for 1.00 ml of column effluent.

The expansion of the solvent–small molecule system as the protein is dissolved in it and the presence of water in the protein sample employed will lower the concentration of small molecule in the initial solution used to dissolve the protein; this will cause the appearance in the elution profile of a trough not resulting from any actual binding. The values of apparent micromoles bound, as determined by either of the above procedures, have been corrected, therefore, by the relationship

$$\text{corrected } \mu\text{moles bound} = \text{apparent } \mu\text{moles bound} - \frac{A_b P[w + \bar{V}(1 - w)]}{\epsilon}$$

where A_b is the base-line absorbance, P is the amount

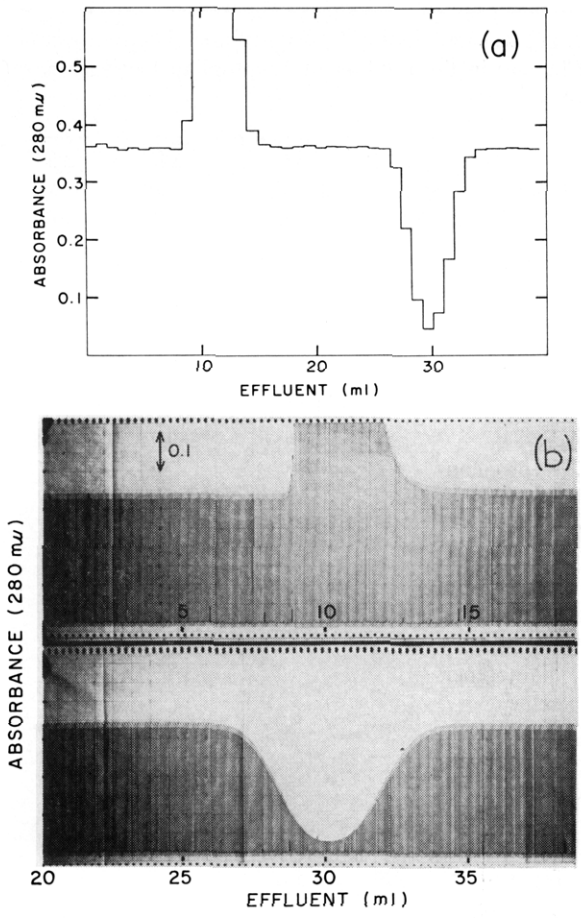

FIGURE 1: Representative elution diagrams for the measurement of the binding of L-tryptophan by BSA. Concentration of L-tryptophan, 6.4×10^{-5} M; amount of protein, 21 mg; pH 9.0; temperature, 30.0°; spectrophotometer cell path length, 1 cm. (a) Elution diagram obtained by analysis of individual fractions of effluent solution. (b) Elution diagram recorded by means of Gilford instrument. In the original record, 5 ml of effluent corresponded to 19.3 cm on the abscissa, and an absorbance of 0.1 corresponded to 5 cm on the ordinate.

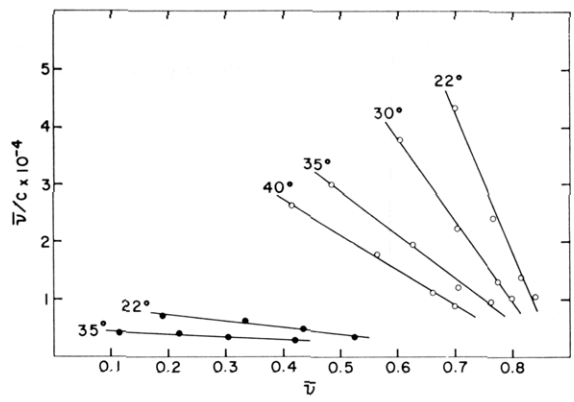

FIGURE 2: Scatchard plots for the binding of L-tryptophan and D-tryptophan by BSA. The amount of protein varied from 10 to 23 mg; pH 9. O, L-tryptophan; ●, D-tryptophan.

of BSA (in milligrams), w is the water content of the BSA sample expressed as a decimal fraction, and $\bar{V}$ is the partial specific volume of the BSA, taken to be 0.74 ml/g. In the case of the strongest binding observed (L-tryptophan) this correction term amounted to less than 1% of the value of apparent μmoles bound. For moderate binding (acetyl-L-tryptophanamide) the correction term was about 5%, and for the weakest binding observed (glycyl-L-tryptophan diketopiperazine) the correction term was about 20% of the value for the apparent micromoles bound.

The data were used for the determination of the value of $\bar{\nu}$, the mean number of moles of small molecule bound per mole of protein. A plot of $\bar{\nu}/c$ against $\bar{\nu}$ gives a straight line with intercepts $k'n$ (as $\bar{\nu}$ approaches zero) and n (as $\bar{\nu}/c$ approaches zero), where k' is the apparent association constant for each of a set of equivalent and independent binding sites on the protein (Scatchard, 1949). In the present work, except where indicated otherwise, the slope of the line (fitted by least squares) through the experimental points was taken as $-k'$. Since the primary purpose of the present study was to examine the utility of the gel-filtration method for the determination of k', no correction was made in our calculations for the influence of salt and of electrostatic effects (Scatchard *et al.*, 1950, 1957; McMenamy and Oncley, 1958; McMenamy, 1964). It will be noted that, in the present work, all experiments were conducted at 0.1 M chloride. McMenamy (1964) has reported that with uncharged indole derivatives (*e.g.*, skatole) changes in the chloride concentration had little effect on the apparent association constant, but that the binding of indole compounds bearing a carboxylate group (*e.g.*, L-tryptophan, acetyl-L-tryptophan) was markedly reduced by increasing the concentration of chloride. A calculation of the Donnan effect on the concentration of acetyl-L-tryptophan in the volume element of the Sephadex column containing the protein indicates that, under our experimental conditions, the presence of protein at a concentration of 20 mg/ml in the external water space can cause a decrease of no more than 2% in the equilibrium concentration of acetyl-L-tryptophan outside the gel particles. Since, in our studies, the protein concentration was usually below this value, no correction was made for the Donnan effect.

Results

In Figure 1a is shown a representative elution pattern for the L-tryptophan–BSA system, obtained by measurement of the absorbance of individual samples of the effluent solution; Figure 1b is a photograph of the elution diagram (from the same experiment) obtained with the Gilford instrument. Although the precision of

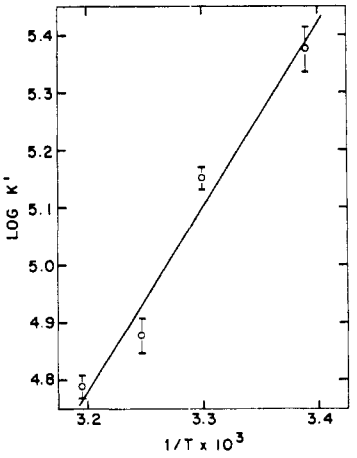

FIGURE 3: Temperature dependence of the binding of L-tryptophan by BSA. The vertical lines at each point denote the standard deviation for that value of k'.

the two methods is similar, the time saved by the latter procedure is considerable.

The results of the gel-filtration studies on the L-tryptophan–BSA system are shown in Figure 2. The pH chosen was 9, because McMenamy and Oncley (1958) had found that the binding of L-tryptophan was maximal near this pH value. At each of the temperatures used (22, 30, 35, and 40°) determinations were made of $\bar{\nu}$ at four concentrations (in the range 1.6×10^{-5} to 7.9×10^{-5} M) of L-tryptophan. The linearity of the $\bar{\nu}/c$ *vs.* plots is satisfactory, and permitted a reliable calculation of k'; the extrapolated values of n vary between 0.85 and 0.88. These results may be considered to be in satisfactory agreement with those of McMenamy and Oncley (1958), whose data gave extrapolated values of n varying from 0.86 to 0.98; they reported that this value was reduced by the addition of oleate or decanol (no effort was made to remove lipids from the BSA preparations used for the gel-filtration studies).

From the data presented in Figure 2, values of k_1' (the apparent association constant for 1:1 interaction) and $\Delta F'$ (the apparent standard free energy change) were calculated for the four temperatures. As will be seen from Figure 3, the plot of log k_1' against reciprocal temperature permitted the calculation of $\Delta H'$ (the apparent standard enthalpy change). The values for k_1' and for the apparent standard free energy, enthalpy, and entropy changes were calculated in the usual manner for 25°, and are given in Table I.

McMenamy and Oncley (1958) reported that bovine mercaptalbumin had essentially no affinity for D-tryptophan at 2° and pH 7.25 or 7.75, and drew attention to the striking stereospecificity in the binding of tryptophan to serum albumin. In subsequent work, McMenamy (1963) found some binding of D-tryptophan by defatted HSA at 18° and pH 9.5, and his data indicate an apparent association constant of 2×10^4 (pH 9.5, 18°). Our gel-filtration studies with D-tryptophan and BSA gave $\bar{\nu}/c$ *vs.* $\bar{\nu}$ plots that were linear, the extrapolated values of n being 0.85 and 0.98 at 22 and 35°, respectively (Figure 2). The association constant (Table I) for the apparent 1:1 interaction of BSA and D-tryptophan is only about one-twentieth the value for L-tryptophan, but is, nevertheless, well within the range of the precision of the gel-filtration method.

In Figure 4 are given data obtained for the binding of acetyl-L-tryptophan by BSA at pH 9 and at pH 6. It will be seen that although the individual points are in reasonable accord with a linear $\bar{\nu}/c$ *vs.* $\bar{\nu}$ plot, the apparent extrapolated value of n is about 1.1. Values of n greater than 1.0 were obtained by McMenamy and Oncley (1958) and by McMenamy and Seder (1963) for acetyl-L-tryptophan and other indole derivatives. Apparent association constants were calculated from the slopes of the lines for the Pentex BSA preparation in Figure 4; the values for k' and the thermodynamic

TABLE I: Association Constants and Thermodynamic Changes in the Binding of Tryptophan Derivatives by BSA.[a]

Compound	k_1' ($M^{-1} \times 10^{-2}$)	$\Delta F'$ (kcal/mole)	$\Delta H'$ (kcal/mole)	$\Delta S'$ (eu/mole)
L-Tryptophan	1910	−7.2	−14.5	−24
D-Tryptophan	93	−5.4	−11.3	−20
Acetyl-L-tryptophan	292	−6.1	− 7.6	− 5
Acetyl-L-tryptophanamide	26	−4.6	−10.0	−18
Acetyl-D-tryptophanamide	19	−4.5		
Glycyl-L-tryptophan-diketopiperazine	2	−3.1		
Glycyl-L-tryptophan	20	−4.5	− 6.8	− 8
Glycyl-L-tryptophanamide	5	−3.4		
L-Tryptophylglycyl-L-tryptophanamide	68	−5.2		
Glycyl-L-tryptophylglycyl-L-tryptophanamide	22	−4.6		

[a] All values are given for 25° and pH 9.0.

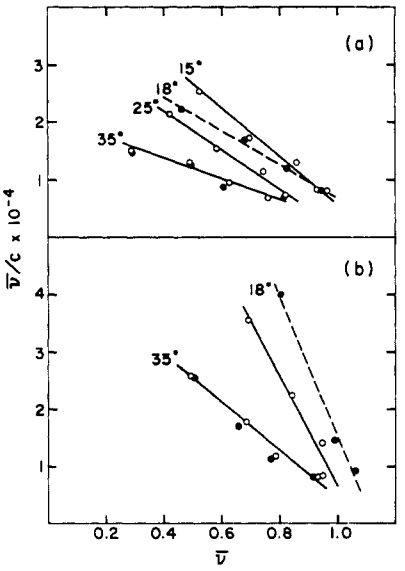

FIGURE 4: Scatchard plots for the binding of acetyl-L-tryptophan by BSA. The amount of protein varied from 7 to 23 mg. O, Pentex BSA (lot No. BX3); ●, Armour BSA (lot No. Y20907). (a) pH 9, (b) pH 6.

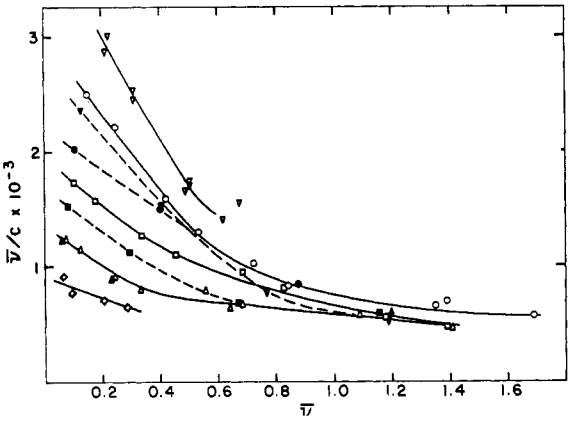

FIGURE 5: Scatchard plots for the binding of acetyl-L-tryptophanamide and acetyl-D-tryptophanamide by BSA. The amount of protein varied between 60 and 70 mg; pH 9. Acetyl-L-tryptophanamide: ▽, 15°; ○, 21°; □, 28°; △, 34°; ◇, 40°. Acetyl-D-tryptophanamide: ▼, 15°; ●, 21°; ■, 28°; ▲, 34°.

parameters given in Table I were calculated on the assumption of a linear temperature dependence of log k', and must be considered as approximate estimates.

The gel-filtration method gives $k_1' = 1.2\ (\pm 0.1) \times 10^5$ for the binding of acetyl-L-tryptophan by the Armour BSA at pH 6 (5.5–6.3), at 0.1 M KCl and 18°. This compares favorably with the value of 1.5×10^5 determined with this preparation by dialysis–equilibrium under the same conditions (Dr. McMenamy, personal communication). These were the only conditions for which a direct comparison could be made as it was not possible, without added buffer, to maintain the system at any other pH in the gel-filtration studies. At pH 9, 0.125 M ethanolamine, 0.1 M Cl^-, and 18°, a value of $k_1' = 3.0\ (\pm 0.3) \times 10^4$ was obtained by gel filtration, and Dr. McMenamy has reported to us that dialysis-equilibrium in 0.1 M KCl at pH 9 and 18° gave a value of 4.0×10^4. These results indicate that both experimental methods give similar results and that ethanolamine does not affect the observed binding greatly, if at all.

It will be noted that, by both experimental methods, and in the presence of 0.1 M KCl, the binding of acetyl-L-tryptophan by BSA is significantly different at pH 6 and 9. This result is in contrast to those obtained with defatted HSA (McMenamy, 1963, 1964); with this protein preparation, the value of k' for the binding of acetyl-L-tryptophan in 0.1 M KCl was found to be essentially constant over the pH range 6–9.

It was of special interest to examine the binding, by BSA, of an uncharged tryptophan derivative such as acetyl-L-tryptophanamide; this compound may be considered to provide a simple model for the behavior of an interior L-tryptophan residue in a linear peptide of appreciable length. As will be seen from Figure 5, the binding of this compound by BSA at pH 9 was slight, but within the range of the precision of the gel-filtration method. Binding experiments were conducted at five temperatures (15, 21, 28, 34, and 40°) in the concentration range 6.1×10^{-5} to 3.0×10^{-3} M, and examination of the $\bar{\nu}/c$ *vs.* $\bar{\nu}$ plots suggests that at low values of $\bar{\nu}$ (0.07 to *ca.* 0.5) the data gave linear plots whose extrapolated intercept on the $\bar{\nu}$ axis is near 0.9; at higher values of $\bar{\nu}$, however, the results are of a kind usually taken to indicate binding to more than one set of sites (McMenamy and Oncley, 1958). We have not attempted to fit a curve to the data shown in Figure 5 by the assumption that, in addition to the single primary binding site n_1 (corresponding to the association constant k_1'), there are an arbitrary number of secondary binding sites n_2. For the purposes of comparison, we have estimated k_1' from the points in the region $\bar{\nu} < 0.5$; the values for log k_1' (at 15, 21, 28, and 34°) when plotted against reciprocal temperature gave a satisfactory straight line, thus permitting the calculation of the magnitude (at 25°) of k_1' and the thermodynamic parameters for the primary association of acetyl-L-tryptophanamide with BSA.

Data are also given in Figure 5 for the binding of acetyl-D-tryptophanamide by BSA at pH 9. It is noteworthy that the sharp stereospecificity observed with L- and D-tryptophan is greatly reduced, and a significant difference in the binding of enantiomorphous acetyltryptophanamides was only observed in these experiments at the lowest temperatures tested. At 34° the points for the D isomer fall on the line drawn through those for acetyl-L-tryptophanamide. In view of the limited data available for the D compound, only approximate estimates can be made of the values of k_1' and $\Delta F'$; these estimates are included in Table I.

Another uncharged derivative of L-tryptophan ex-

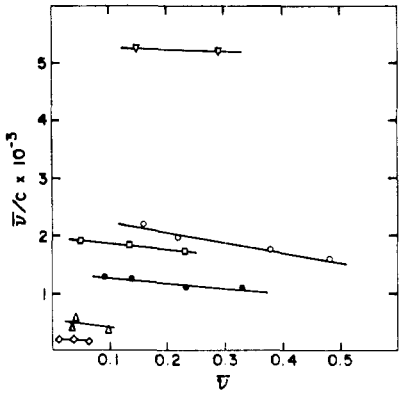

FIGURE 6: Scatchard plots for the binding of tryptophan peptides by BSA. The amount of protein varied from 30 to 40 mg; pH 9. ○, glycyl-L-tryptophan, 15°; ●, glycyl-L-tryptophan, 30°; ◇, glycyl-L-tryptophan diketopiperazine, 25°; △, glycyl-L-tryptophanamide, 25°; ▽, L-tryptophylglycyl-L-tryptophanamide, 25°; □, glycyl-L-tryptophylglycyl-L-tryptophanamide, 25°.

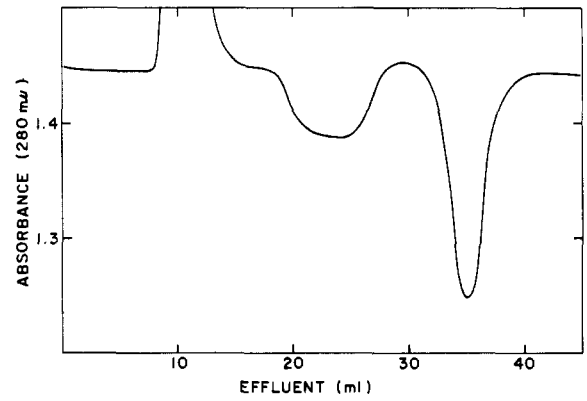

FIGURE 7: Representative elution diagram for the measurement of the competition between acetyl-L-tryptophan and acetyl-L-tryptophanamide for binding by BSA. This diagram is a copy of that recorded by means of the Gilford instrument. Amount of BSA, 20 mg; concentration of acetyl-L-tryptophan, 1.54×10^{-5} M; concentration of acetyl-L-tryptophanamide, 2.68×10^{-4} M; pH 9; temperature, 15°; spectrophotometer cell path length, 1 cm. The first trough after the protein peak corresponds to acetyl-L-tryptophan, the second to acetyl-L-tryptophanamide.

amined for its capacity to be bound by BSA was glycyl-L-tryptophan diketopiperazine. Data were obtained at 25° (Figure 6) and showed that the cyclic dipeptide is bound very weakly by the protein, the estimated value of k_1' being 200, or an order of magnitude less than that found for acetyl-L-tryptophanamide.

It was of considerable interest to find that glycyl-L-tryptophan is bound at pH 9 much less firmly than either L-tryptophan or acetyl-L-tryptophan; the pH dependence of the binding of the dipeptide was not examined, and remains for future study. Data were obtained at four concentrations of glycyl-L-tryptophan to give linear plots for 15 and 30° (Figure 6); from the slopes, k' was estimated to be 1.7×10^3 ($n = 1.4$) and 8.8×10^2 ($n = 1.5$), respectively. The values of k_1' were estimated by making the assumption that the maximal binding at the primary tryptophan site of our BSA preparation is 0.9; by use of the lowest values of $\bar{\nu}$ (*ca.* 0.1–0.2), the k_1' values obtained were 2.9×10^3 and 1.6×10^3, respectively. On the assumption of a linear temperature dependence of k_1' in the range 15–30°, its value at 25° was estimated, as were the corresponding values for the thermodynamic parameters in the binding of the dipeptide to BSA (Table I).

Binding studies with glycyl-L-tryptophanamide, L-tryptophylglycyl-L-tryptophanamide, and glycyl-L-tryptophylglycyl-L-tryptophanamide were performed at pH 9 and 25°. Although insufficient data were obtained to characterize fully the binding curves for each of these compounds, some conclusions can be drawn about their relative ability to be bound to BSA. It can be seen from Figure 6 that the dipeptide amide is bound less strongly than the corresponding dipeptide, whereas the introduction of a second tryptophyl group to form the tripeptide amide, L-tryptophylglycyl-L-tryptophanamide, leads to a 10-fold increase in the value of the association constant (Table I). On the other hand, the addition of an N-terminal glycine to form the tetrapeptide amide, glycyl-L-tryptophylglycyl-L-tryptophanamide, results in a decrease of the affinity between the peptide and the protein.

The gel-filtration method appears well suited to the quantitative study of the competition of two small molecules for binding by a protein. An essential requirement is that the column elution volumes of the competing species be sufficiently different for the troughs in the elution diagram to be sufficiently separated, or that different analytical methods are available for the separate determination of the values of c for each species. In the dialysis–equilibrium studies of McMenamy and Oncley (1958) and McMenamy and Seder (1963), the components of the dialysate were separated by paper chromatography, and visual comparison was made of the intensity of color given by Ehrlich's reagent with that of a series of standards.

As will be seen in Figure 7, the competition between two tryptophan derivatives for binding by BSA has been studied directly for the case of the competition between acetyl-L-tryptophan and acetyl-L-tryptophanamide. If the two compounds compete for the same primary binding site on the albumin molecule, the following relationship should hold (Klotz *et al.*, 1948)

$$\frac{\bar{\nu}_A}{\bar{\nu}_B} = \frac{k_{1A}' \times c_A}{k_{1B}' \times c_B}$$

where $\bar{\nu}_A$ is the amount of acetyl-L-tryptophan bound in the presence of a given amount of acetyl-L-tryptophanamide; $\bar{\nu}_B$ is the amount of acetyl-L-tryptophan-

amide bound in the presence of a given amount of acetyl-L-tryptophan; k_{1A}' is the apparent primary binding constant for acetyl-L-tryptophan; k_{1B}' is the apparent primary binding constant for acetyl-L-tryptophanamide; c_A is the concentration of acetyl-L-tryptophan; c_B is the concentration of acetyl-L-tryptophanamide. This relationship was tested by determining the amount of each compound bound at a constant level of acetyl-L-tryptophanamide ($k_{1B}' = 4.5 \times 10^3$, pH 9, 15°) and varying levels of acetyl-L-tryptophan ($k_{1A}' = 4.3 \times 10^4$, pH 9, 15°). The values found for $\bar{\nu}_A/\bar{\nu}_B$ by the gel-filtration technique are in reasonably good agreement with those calculated by means of the above formula (Table II). This concordance supports the

TABLE II: Competition of Acetyl-L-tryptophan and Acetyl-L-tryptophanamide for Binding by BSA.[a]

Concentration of Acetyl-L-tryptophan (M)	$\bar{\nu}_A/\bar{\nu}_B$ Calcd	$\bar{\nu}_A/\bar{\nu}_B$ Obsd
1.54×10^{-5}	0.55	0.60
2.31×10^{-5}	0.82	0.91
3.08×10^{-5}	1.10	1.30

[a] The experiments were performed at 15° and pH 9. The concentration of acetyl-L-tryptophanamide was 2.68×10^{-4} M.

view that acetyl-L-tryptophan and acetyl-L-tryptophanamide are both bound by BSA at the same primary binding site. Since McMenamy and Oncley (1958) showed that L-tryptophan and acetyl-L-tryptophan compete for the same primary binding site, it may be inferred that the primary binding site of acetyl-L-tryptophanamide is at the tryptophan site of BSA.

In studying peptide–protein interactions where the association constant was relatively small, it was necessary to use relatively large amounts of protein in the gel-filtration experiments. As indicated in the Experimental Section, the protein was applied to the column in 1.0 ml of solution, and it was important to ascertain whether the value of $\bar{\nu}$ changed wih the protein concentration in this solution. The data in Table III indicate that, over the range of amounts of BSA used in the experiments reported in this communication (0.1–1.0 μmole or 7–70 mg), there was no significant variation of the value for $\bar{\nu}$ in the binding of acetyl-L-tryptophanamide. It may be added that, in all cases, the protein emerged from the column in essentially the same volume of effluent (4–5 ml).

In all the gel-filtration experiments described above the amount of small molecule bound at equilibrium was approached from a lower free concentration in the environment of the protein. If the technique allows the attainment of equilibrium, the amount of small molecule bound should be the same when the equilibrium

TABLE III: Dependence of Binding of Acetyl-L-tryptophanamide on Amount of BSA.[a]

Run	Acetyl-L-tryptophanamide (M × 10⁵)	BSA applied in 1 ml (μmoles)	$\bar{\nu}$
1a	4.79	0.299	0.066
1b	4.80	0.584	0.064
2a	9.70	0.291	0.111
2b	9.72	0.590	0.112
3a	29.3	0.510	0.502
3b	28.9	0.810	0.502
3c	29.3	0.831	0.487

[a] Runs 1a, 1b, 2a, and 2b were performed with Pentex BSA lot No. 9G10, in 0.1 M glycine buffer pH 9.0, 30°; runs 3a, 3b, and 3c were performed with Pentex BSA lot No. BX3, 0.125 M ethanolamine buffer, pH 9.0, 15°.

is approached from a higher free concentration of small molecule. This approach from a higher free concentration was accomplished by dissolving BSA (*ca.* 1 μmole) in 1.0-ml solutions containing an amount of acetyl-L-tryptophanamide in excess of that required for binding at equilibrium. The amount of small molecule bound by the protein at a given level of small molecule was then determined by the difference between effluent peaks of small molecule in the presence and absence of protein. In one experiment, 0.28 μmole of acetyl-L-tryptophanamide was present in the 1 ml of protein solution (0.19 μmole required for binding) and the concentration of the small molecule in the eluting solution was 7.4×10^{-5} M. In another experiment, 0.41 μmole of acetyl-L-tryptophanamide was present initially (0.26 μmole required for binding) and the concentration of small molecule in the eluting solution was 12.5×10^{-5} M. Both experiments were performed at 15° and pH 9. The approach from above equilibrium levels gave $\bar{\nu}$ values of 0.224 and 0.310, respectively, and the usual approach from below gave values of 0.210 and 0.311, respectively.

Discussion

The data presented above indicate that the gel-filtration method may be the technique of choice for the determination of association constants in the interaction of proteins (and other macromolecules) with smaller compounds. As pointed out by Hummel and Dreyer (1962), the principal requirement for the use of the method is that the type of gel chosen excludes the larger of the interacting species; clearly, precise analytical procedures are needed for the determination of the concentration of the smaller molecule in the effluent. Although spectrophotometry is most convenient when possible, other techniques (polarimetry, determination

of radioactivity, specific chemical reactions, etc.) could also be applied in an automated procedure.

Further work is needed to permit a critical assessment of the precision of the gel-filtration method in relation to that of other procedures, such as dialysis equilibrium. The data available for the L-tryptophan–BSA system suggest, however, that the gel-filtration method is at least as precise as dialysis equilibrium, and that it offers decided advantages in terms of speed, convenience, and flexibility. In many cases, the gel-filtration method may be better suited for the study of the competition of two small molecules for a binding site on a protein. For example, it may be usefully applied in the competition method recently described by Weiner and Koshland (1965).

An essential criterion for the attainment of equilibrium in the operation of the gel-filtration method is the return of the base-line concentration of the small molecule to its original level after the emergence of the protein and after the appearance of the trough in the elution diagram. The data on the binding of L-tryptophan and of acetyl-L-tryptophanamide by BSA indicate that equilibrium conditions can be met satisfactorily in the gel-filtration method, and that for simple 1:1 interaction of a protein with a small molecule the data have theoretical validity. This is shown by the results in Figures 1–3 for the L-tryptophan–BSA system, by the experiment with acetyl-L-tryptophan and acetyl-L-tryptophanamide as competitors for binding with BSA, and by the experiment in which the protein had been equilibrated with excess acetyl-L-tryptophanamide before being applied to the column.

It was gratifying to find essential agreement between our data for k_1' and those of McMenamy and his associates, when a meaningful comparison could be made. In view of the sensitivity of the L-tryptophan–BSA interaction to various experimental factors (pH, temperature, ionic strength, effect of solvent ions, state of the protein, etc.), the concordance of the results obtained by gel filtration and dialysis equilibrium appears to be satisfactory. Whether similar agreement will be found for other protein–small molecule interactions as studied by the two methods remains to be determined in future work.

Comparison of the binding constant for the L-tryptophan–BSA system with those found for compounds related to L-tryptophan led McMenamy and his associates to draw several conclusions regarding the effect of structural changes in the small molecule on its binding by serum albumin. The most noteworthy feature was the sharp stereospecificity for the tryptophan enantiomers (McMenamy and Oncley, 1958; McMenamy, 1963); this has been confirmed in the present work. McMenamy and Oncley (1958) also noted a significant difference in the binding of the methyl esters of L- and D-tryptophan (the association constant for L-tryptophan was only about four times the value for L-tryptophan methyl ester), suggesting that the carboxylate group may not be essential for binding at the tryptophan site of BSA. The effect of pH on the binding of L-tryptophan and of tryptamine (McMenamy and Seder, 1963; McMenamy, 1964) indicates that protonation of the α-amino group inhibits binding. The fact that indole propionate, acetyl-L-tryptophan, and skatole are strongly bound by HSA (McMenamy and Oncley, 1958; McMenamy, 1964) shows, however, that the unprotonated amino group is not essential for binding; indeed, it would appear that indole propionate is bound to HSA more strongly than is L-tryptophan. The data of McMenamy and Seder (1963) on the competition between indole propionate and L-tryptophan suggest that these two substances compete strongly for a primary binding site, but less strongly for secondary binding sites.

It is clear from the work of many investigators (see Klotz, 1953; Steinhardt and Beychok, 1964) that serum albumin has a large number of potential binding sites for anions, and extensive evidence has been presented for the interaction of organic anions with cationic groups on the protein. That the anion-binding sites of HSA do not behave identically in their interaction with small ions is evident from the recent studies of Scatchard and Yap (1964). These investigators have shown, for example, that in the binding of iodide by HSA, there is one very active site, whose interaction with the anion is associated with a large enthalpy change and a negative entropy change; the weaker binding at the secondary sites is associated with a positive entropy change.

As a first approximation, it may be postulated that one of the anion-binding sites of BSA is suitably located in relation to a binding site for the indolylmethylene side chain of tryptophan. (The results of McMenamy and Oncley (1958) with chemically modified serum albumin suggest that the terminal α-amino group of the protein may provide a cationic group involved in the 1:1 binding of L-tryptophan.) If this assumption proves to be correct, the region of the BSA molecule involved in the specific binding of L-tryptophan may be considered to have at least two interacting binding sites. To explain fully the stereospecificity of the interaction, it seems necessary to assume an additional structural constraint. One possibility is the requirement that a group no larger than a hydrogen atom be situated at the locus of the α-hydrogen of L-tryptophan; this is supported by the observation of McMenamy and Oncley (1958) that α-methyltryptophan is bound very poorly by BSA, if at all. More extensive speculation about the geometry of the tryptophan-binding site of serum albumin appears unwarranted at present. Any model of this binding site must take into account, however, the relatively poor binding of glycyl-L-tryptophan diketopiperazine, as compared to skatole or acetyl-L-tryptophanamide, as well as the small but significant stereospecificity for the binding of the enantiomers of acetyltryptophanamide at low temperatures. A similar small difference in the binding of the enantiomers of an organic dye has been found by Karush (1954). It may be added that Markus (1965) has observed a decrease in the rate of proteolysis of HSA in the presence of 0.001 M L-tryptophan, and has attributed this result to the stabilization of a particular conformational state as a

consequence of the 1:1 binding of the amino acid.

The question of the possible role of hydrophobic bonding in the interaction of BSA with anionic organic dyes and detergents has been discussed by many investigators. Kauzmann (1959) has suggested that the positive entropy change found in such interactions (*e.g.*, Klotz and Urquhart, 1949) may be a consequence of the transfer of apolar groups from the aqueous phase to an apolar environment within or on the protein. The data in Table I indicate that there is a sizeable negative entropy change in the binding not only of L-tryptophan, but also of the uncharged acetyl-L-tryptophanamide. This raises the question whether the mode of binding of L-tryptophan and its derivatives to the tryptophan-binding site of BSA is different in nature from that in the binding of anionic organic dyes. This question must be left open for further study, however, in view of the different values of the enthalpy change recorded for the binding of L-tryptophan by serum albumin. McMenamy and Seder (1963) reported a value of −2.3 kcal/mole for HSA, and McMenamy (1963) gave a value of −10.9 kcal/mole for defatted HSA; the latter value is near that given in Table I for BSA (−14.5 kcal/mole). In this connection, it may be noted that the thermodynamic data given in Table I for the binding of acetyl-L-tryptophan by BSA are in reasonable agreement with the values reported by McMenamy (1964). The sizeable enthalpy changes recorded in Table I are greater than those reported for the binding of organic anions to BSA (Kauzmann, 1959).

That the primary interaction of the tryptophan derivatives with BSA may not be solely a consequence of hydrophobic bonding arising from the unfavorable interaction of apolar groups with water is suggested by the relatively low association constants for the tripeptide amide and tetrapeptide amide tested (each contains two tryptophyl residues). To what extent this low binding is caused by an inhibitory effect of the terminal amino group cannot be stated; the corresponding N-substituted compounds were insufficiently soluble in aqueous solvents for the determination of their binding by BSA.

The fact that the primary association constant for acetyl-L-tryptophanamide (Table I) is less than that for skatole (McMenamy, 1964) indicates that the CO–NH groups of the small molecule do not make a significant contribution to the interaction with BSA. This is in accord with the conclusion of Klotz and Franzen (1962) that hydrogen bonds formed in the interaction of CO–NH groups with water are much stronger than hydrogen bonds between CO–NH groups. The biological significance of the binding of L-tryptophan by serum albumin has been discussed by McMenamy *et al.* (1961). Rubin *et al.* (1963) have drawn attention to the association of peptide-like material with the albumin fraction of human plasma.

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