The Specific Binding of L-Tryptophan to Serum Albumin*

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In a recent study of recovery of amino acids added to human plasma, it was found that L-tryptophan was bound by a non-dialyzable plasma component (1). In the present work, this solute is identified as serum albumin. This observation is of considerable interest since tryptophan is an essential amino acid, with many pathways of metabolism. An investigation was, therefore, undertaken to determine which structural features of tryptophan are responsible for its binding and to elucidate the nature of the binding site of the protein.

EXPERIMENTAL

Materials

Proteins—The proteins used in this study were prepared without contact with small organic molecules except citrate, dextrose, ethanol, and acetate used in blood collection and fractionation.

Fresh human plasma fractions were obtained from the Protein Foundation, Jamaica Plain, Massachusetts. Each was dialyzed extensively against buffer before studies of binding were undertaken. Human Fraction V was estimated by electrophoretic analysis to be 97 per cent albumin and 3 per cent α-globulin. It was dialyzed extensively against 0.1 M NaCl solution and then against water; then it was deionized by the method of Dintzis (2). Bovine Fraction V, obtained from Armour Laboratories, Chicago, Illinois, was dialyzed extensively against buffer before use. Bovine mercaptalbumin (crystallized), prepared by the method of Dintzis (2), was kindly supplied by Dr. T. Thompson, formerly of this laboratory.

Preparation of Guanidinated Albumin—Bovine mercaptalbumin was guanidinated by the method of Hughes et al. (3). The extent of guanidination was controlled by pH and quantity of O-methylisourea added. In Table I the conditions used for each preparation are listed. The amount of guanidination was determined by amino acid analyses (see below). Ultracentrifugal analysis of one preparation, 5CII, indicated no gross heterogeneity in molecular weight.

Preparation of Acetylated Albumin—The method of Frankei-Conrat et al. (4) was used for acetylation of bovine mercaptalbumin. The extent of reaction was controlled by the amounts of acetic anhydride added. Table I gives the conditions used for these preparations. Amino acid analyses performed on the hydrolysates were used to determine the groups acetylated. Ultracentrifugal analysis of one preparation, 135CI, indicated no gross heterogeneity in molecular weight.

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Acetylation of Guanidinated Albumin—Guanidinated albumin preparations 5AI and 5CII were acetylated under the conditions specified in Table I. The groups acetylated were determined by paper chromatography.

Tryptophan and Its Homologues—L-Tryptophan and D-tryptophan were obtained from Mann Research Laboratories, Inc., New York. Each was recrystallized twice from hot 70 per cent methanol, as suggested by Beaven and Holiday (5). $\alpha^{[\text{H}]_{14}}$ found for L-tryptophan was $-36.5^\circ$, and for D-tryptophan, $+35.0^\circ$. N-acetyl-L-tryptophan, L-tryptophan ethyl ester, tryptamine, and 5-hydroxytryptamine were obtained from Mann Research Laboratories, Inc. These compounds were not recrystallized.

α-Methyl DL-tryptophan, DL-tryptazan, 5-methyl DL-tryptophan, and 6-methyl DL-tryptophan were kindly supplied by Dr. N. Tanaka and Dr. Eugene Knox of the Cancer Research Institute, New England Deaconess Hospital, Boston. The 5- and 6-methyl derivatives had been prepared by Dr. H. R. Snyder (6). L-Tryptophan methyl ester and D-tryptophan methyl ester were prepared by dissolving α- or β-tryptophan in cold absolute methanol ($-5^\circ$) saturated with hydrogen chloride.

Guanidinated L-tryptophan (β-indole-α-guanidopropionic acid) was prepared by the method of Kapfhammar and Müller (7) with the use of O-methylisourea and tropphan.

Other Reagents—O-methylisourea hydrochloride was prepared by the method of McKee (8). Cyanamide for this preparation was obtained from the Fisher Scientific Company, New York. 2-Amino-2-hydroxymethyl-1,3-propanediol, was obtained from Sigma Chemical Company, St. Louis.

Methods

Dialysis—Binding was studied by equilibrium dialysis. 30 feet of Visking dialysis tubing, size 23/32, were washed, to remove associated impurities, by soaking in frequently changed distilled water (added through the inside of the tubing) for 3 days. The dialysis tubing was then cut into 1-foot lengths; each length was tied at one end, and after a sealed glass tube (50 mm. long, 16 mm. outside diameter) partly filled with lead shot had been placed inside, was inflated gently with nitrogen and clamped shut. When dry, the membranes were cut beneath the clamp and inserted into glass tubes containing 4 ml. of 0.1 M NaCl buffer solution. The glass tubes were of 220-mm. length and 20-mm. inside diameter and had the tips drawn to a point to accommodate the knot of the membrane. Inside the dialysis sacs were placed 2.5 ml. of an approximately 2.8 per cent protein solution. The small molecule whose binding to albumin was to be measured was added to either the inside or the outside solution. The end of the dry dialysis tubing extended well above the solution levels and was left open. A maximum of 30 such
dialysis tubes was then placed on a rotating rack constructed to hold the tubes at an angle of 30° to the horizontal and to rotate them at 5 r.p.m. The weighted glass tubes inside the dialysis sacs gave a thin layer of protein solution, and, as the dialysis tubes rotated, agitated both the dialysate and protein solution by pressing against the downward side. Equilibrium was obtained in 5 hours at 2°C.

**Determination of Tryptophan and Its Homologues**—The concentrations of tryptophan and its homologues were determined in the dialysates by one of two methods as follows.

1. **Spectrophotometric**. Absorptions of solutions of the compounds were measured in the ultraviolet region (Table II) with the use of a Beckman model DU spectrophotometer. To account accurately for background, each set of experiments was performed with three or more control tubes containing protein solution, salt solution, and the membrane, but none of the binding molecule. The average optical density of the control dialysates was subtracted from each analysis. Background readings from tubing of the same washing showed a variability equivalent to a standard deviation of 0.0013 mM tryptophan. At concentrations greater than 0.03 mM tryptophan the coefficient of variation of an analysis was estimated at 5 per cent. Negligible amounts of the small molecules were found to be bound to the membrane.

2. **Paper chromatography**. In competitive studies between indole compounds it was necessary to separate the compounds to measure their concentrations. This was accomplished by one-dimensional paper chromatography as follows.

Solvent A was the buffered, salt-saturated, isopropanol solvent A described by McMenamy et al. (1); the papers were pretreated according to the method reported in the same reference. The time of development on Whatman No. 1 paper was reduced to 6 hours. Competitive studies of L-tryptophan (RF = 0.35) with L-tryptophan ethyl ester (RF = 0.85), D- and L-tryptophan methyl esters (RF = 0.75), and N-acetyl-L-tryptophan (RF = 0.70) were analyzed with this solvent.

Solvent B was an acetic acid, water, and isopropanol mixture (volume ratios, 10:20:70) saturated with salt by addition of solid sodium chloride. Whatman No. 1 paper, dried after being dipped in a 3 per cent aqueous solution of sodium chloride, was used for the chromatograms. The chromatograms were developed for 10 hours. Solvent B was used for the competitive studies of L-tryptophan (RF = 0.70) with 5-methyl DL-tryptophan (RF = 0.75), 6-methyl DL-tryptophan (RF = 0.80), α-methyl DL-tryptophan (RF = 0.80), and DL-tryptazan. The RF value of DL-tryptazan was not determined since it gave no color reaction with the indole staining reagent.

The quantity of the indole compounds in the dialysates was determined as follows. An aliquot of each dialysate was concentrated 30-fold by freeze-drying and applied to the paper in 3-, 6-, and 12-μl. volumes. A standard solution containing tryptophan (1 mM) and the competitive indole derivative (1 mM) were placed on the paper in 1.5-, 3-, 6-, 9-, and 12-μl. volumes. After the chromatograms had been developed and dried, they were stained with Ehrlich's reagent according to Smith's modification (9). The amounts of each indole compound on the paper were estimated by visual comparison of its zone color intensity with that of the standard. The coefficient of variation in this determination was approximately 6 per cent.

**Analysis of Protein Hydrolysates**—Two hydrolysates were performed, one directly on the modified albumin, and the other on the modified albumin after it had been treated with dinitrofluorobenzene by Brown's procedure (10) (DNP-modified albumin).

**Hydrolysis**. The proteins were hydrolyzed in evacuated, sealed glass tubes at 110°C for 16 hours with the use of 6 N HCl. The hydrolysates were dried and dissolved in 0.5 M NH₄OH in the ratio of 0.5 ml./8 mg. of protein hydrolyzed for amino acid analyses and in the ratio of 0.2 ml./20 mg. of protein hydrolyzed for DNP-aspartic acid analyses.

**Standards**. Hydrolysates of the original albumin, and of the original albumin after reaction with dinitrofluorobenzene, were prepared under the same conditions used for the modified pro-

**Table I**

<table>
<thead>
<tr>
<th>Protein modifications†</th>
<th>Experiment No.</th>
<th>Alba-</th>
<th>Reagent</th>
<th>Volume</th>
<th>pH</th>
<th>Time</th>
<th>α-NH₂</th>
<th>ε-NH₂</th>
<th>Percentage of groups modified</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>min.</td>
<td>µoles</td>
<td>µoles</td>
<td>ml.</td>
<td>days</td>
<td></td>
<td></td>
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<tr>
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<td>4,800</td>
<td>2</td>
<td>10.5</td>
<td>3</td>
<td>0</td>
<td>95</td>
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<tr>
<td></td>
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<td>4,500</td>
<td>2</td>
<td>8.5</td>
<td>4</td>
<td>0</td>
<td>65</td>
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<tr>
<td></td>
<td>5CII</td>
<td>6.6</td>
<td>4,500</td>
<td>2</td>
<td>9.5</td>
<td>4.5</td>
<td>10</td>
<td>70</td>
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<tr>
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<td>8.0</td>
<td>3</td>
<td>50</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>135CI</td>
<td>6.6</td>
<td>145</td>
<td>2</td>
<td>8.0</td>
<td>3</td>
<td>80</td>
<td>17</td>
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</tr>
<tr>
<td></td>
<td>135I</td>
<td>6.6</td>
<td>290</td>
<td>2</td>
<td>10.0</td>
<td>3</td>
<td>100</td>
<td>35</td>
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<td>68</td>
<td></td>
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<tr>
<td></td>
<td>6AII</td>
<td>2.2</td>
<td>25</td>
<td>2</td>
<td>8.0</td>
<td>5</td>
<td>&gt;100</td>
<td>78</td>
<td></td>
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</table>

† Modifications were made at 2°C. Dialysis was used to remove unreacted reagents.

**Table II**

<table>
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<th>Substance</th>
<th>Molar extinction coefficient</th>
<th>Wave length</th>
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<tr>
<td>L-Tryptophan</td>
<td>5650</td>
<td>279</td>
</tr>
<tr>
<td>D-Tryptophan</td>
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<td>279</td>
</tr>
<tr>
<td>Acetyl-L-tryptophan</td>
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<td>279</td>
</tr>
<tr>
<td>L-Tryptophan ethyl ester</td>
<td>5650</td>
<td>279</td>
</tr>
<tr>
<td>L-Tryptophan methyl ester</td>
<td>5650</td>
<td>279</td>
</tr>
<tr>
<td>n-Tryptophan methyl ester</td>
<td>5650</td>
<td>279</td>
</tr>
<tr>
<td>Tryptamine</td>
<td>5300</td>
<td>279</td>
</tr>
<tr>
<td>5-Hydroxytryptamine</td>
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<td>275</td>
</tr>
<tr>
<td>Indoleacetic acid</td>
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<td>279</td>
</tr>
<tr>
<td>3-Hydroxyindoleacetic acid</td>
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<td>279</td>
</tr>
<tr>
<td>α-Methyl DL-tryptophan</td>
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<td>279</td>
</tr>
<tr>
<td>6-Methyl DL-tryptophan</td>
<td>5650</td>
<td>279</td>
</tr>
<tr>
<td>5-Methyl DL-tryptophan</td>
<td>5650</td>
<td>279</td>
</tr>
<tr>
<td>DL-Tryptazan</td>
<td>5650</td>
<td>288</td>
</tr>
<tr>
<td>Guanidinated L-tryptophan</td>
<td>5000</td>
<td>280</td>
</tr>
</tbody>
</table>

The abbreviation used is: DNP-, dinitrophenyl-.
The two hydrolysates were used as standards to determine the percentage of the amino acids which had been modified in the acetylated and guanidinated preparations.

**Analysis for DNP-Aspartic Acid**—The N-terminal amino acid residue of human and bovine serum albumin is aspartic acid (10-12). The extent to which the α-amino group of aspartic acid was acetylated or guanidinated was determined by analysis for DNP aspartic acid in hydrolysates of DNP modified proteins. Because of the destruction of DNP-aspartic acid during hydrolysis the amount present was determined relative to the control hydrolysate of unmodified albumin reacted with dinitrofluorobenzene. The amount of DNP-aspartic acid (RF = 0.10) was determined by elution of a series of zones from a row of 10 applications (5 μl each) on Whatman No. 1 paper developed for 4 hours in Solvent A, and by reading the optical density of the eluate at 360 μm (13).

**Analysis to Determine Amounts of Lysine Modified**—The hydrolysates of modified albumins, and modified albumins after reaction with dinitrofluorobenzene were analyzed for free lysine. 3-, 6-, and 12-μl aliquots were placed on S and S No. 589 paper (green ribbon) which had previously been dipped in a borate buffer solution, pH 8, containing 3 per cent sodium chloride and then dried (1). As a standard, the hydrolysate of unmodified albumin was placed on each paper in 1-, 2-, 3-, 6-, and 12-μl aliquots. The chromatograms were developed in the isopropanol, salt-saturated Solvent A for 60 hours. They were dried and stained with a ninhydrin-collidine reagent (1). The amounts of lysine present in each were visually estimated relative to the standard.

In the guanidinated preparations, the value of lysine found in the hydrolysates of the protein untreated with dinitrofluorobenzene was taken as the fraction of lysine which had not reacted with O-methylisourea. In acetylated preparations the amounts of lysine found in the DNP-protein hydrolysates were equivalent to the fraction of lysine acetylated.

**Analysis to Determine Amounts of Protein Hydrolyzed**—The amount of protein which had been placed in the sealed tubes for hydrolysis was determined from the analysis of three amino acids, viz. alanine, arginine, and glutamic acid. These analyses were made from the same chromatograms used for lysine determinations. The composite average value of these three amino acids was used as a parameter to adjust all concentrations relative to the standard.

**pH Measurements**—After dialysis, hydrogen ion activities of the protein solutions were measured at 2° after dilution to an approximate concentration of 0.7 per cent protein with 0.1 M NaCl. The pH meter used was manufactured by the Cambridge Instrument Company, Inc., of Ossining, New York. In a few instances the hydrogen ion activities of unbuffered protein solutions were measured at room temperature and converted to 2° by use of Tanford’s titration data (14) assuming that ΔΗ was constant over the temperature range of 0-25°.

**Measurement of Protein Concentrations**—Concentrations of protein were determined by refractive index measurements (15). Concentrations of some of the modified albumins were also determined spectrophotometrically at 250 μm, with the use of extinction coefficients of 5.3 and 6.6 for 1 per cent solutions of human and bovine albumin, respectively (16).

**Equations and Experimental Factors Used in this Study**

1. **Equations**—Equation 1, given by Scatchard (17), is the general equation which takes into account the statistical and electrostatic effects in association of small molecules to multiple sites, n, of the same intrinsic affinities, k. on a protein.

\[ \frac{\varphi}{a} \varphi^2 \varphi^2 = k \varphi^2 \]  

\[ (B) \]

where \( \varphi \) is the molar ratio of the bound small molecule to albumin, A is the molar concentration of the small binding molecule, \( Z_a \) and \( Z_A \) are the net charges on the protein molecule and small molecule, respectively, and \( w \) is the electrostatic parameter of the Debye-Hückel theory. The electrostatic term, \( \varphi^2 \varphi^2 \), equals 1 when the small molecule has zero net charge. A plot of \( (\varphi/A)(\varphi^2 \varphi^2) \) versus \( \varphi \) gives a straight line with intercepts \( k_0 \) (as \( \varphi \rightarrow 0 \)) and \( n \) (as \( \varphi/A \)).

2. **Experimental Parameters Used**—A molecular weight of 69,000 was assumed for bovine and human serum albumin. The net charge on the protein, \( Z_a \), was taken from Tanford’s titration curve at 0° (14) with an additional 5 negative charges added to take into account chloride-binding in 0.1 M NaCl solution. The binding was approximated by extrapolation of the data of Scatchard et al. (18). The electrostatic parameter, \( w \), was calculated from molecular dimensions, using 30 Å for the radius of the albumin molecule and 32.5 Å as the radius of exclusion. A partial specific volume correction was made, allowing 0.74 ml per gm of albumin, for the solution inside the dialysis sac.

**RESULTS**

**Binding of L-Tryptophan to Albumin at pH 7.4**

L-Tryptophan did not bind appreciably to any plasma fraction at pH 7.4 unless the latter contained albumin. Fig. 1 shows the binding of L-tryptophan to bovine mercaptalbumin in 0.05 M phosphate buffer at pH 7.4 and 2°. The experimental points were best fitted by a binding constant of 1.6 × 10^10 and a value of n, the number of binding sites on the albumin molecule, equal to 0.94. Since this was a protein preparation of high purity, the values indicated that less than one site per albumin molecule was available for binding. Any correction which allowed for loss of tryptophan further reduced...
this value. It was thus suspected that a maximum of one site on the albumin was binding, but that this one site was partially blocked. (See below, "Effects of Fatty Acids on Binding of L-Tryptophan.") With human albumin, an n value of slightly less than 1 was similarly found.

**pH Dependence of L-Tryptophan-binding**

The binding of L-tryptophan to Fraction V from human plasma was studied as a function of pH in 0.1 M NaCl. In Fig. 2 it is seen that a change of pH did not change the number of sites at which the strongest binding occurred. There was, however, a small amount of binding at other positions as indicated by v values greater than 1 at high concentrations of tryptophan (data not shown). This secondary binding was of a very low order of magnitude. At pH 9.2 it was estimated that this binding constant was not greater than 500 for a minimum of 10 sites.

The manner in which binding varies with pH can be better seen in Fig. 3. In this figure, k', the observed association constant at different pH values, was plotted versus pH. A maximum occurred at about pH 9.2. On either side of this maximum, k' decreased rapidly. The decrease in binding below pH 9.2 could not be attributed to a change in the charge of tryptophan, since it is primarily in the form of a dipolar ion of zero net charge over this pH range. It thus seemed that the drop in binding affinity was attributable to a change on the protein, presumably caused by the association of a proton on a group in the vicinity of the binding site. Above pH 9.2 the decrease in binding affinity could be accounted for in several ways. The most likely explanation appeared to be that it was ascribable to electrostatic repulsive forces between the protein and the anionic form of tryptophan. The amino group of tryptophan was found by means of titration to have a pK' of 10.06 at 2° in 0.1 M NaCl. Other effects, such as a low intrinsic binding constant for the anionic form of tryptophan or a change in ionization of a group on the protein, could also lead to a decrease in binding in the higher pH region.

Let the following equations represent the molar concentrations of the various forms of the molecules available for association in binding complexes:

\[ T^+ + H_2NR\beta_p \rightleftharpoons T^+H_2NR\beta_p \]  
\[ T^- + H_2NR\beta_p \rightleftharpoons T^-H_2NR\beta_p \]  
\[ T + H_2NR\beta_{p-1} \rightleftharpoons T\times H_2NR\beta_{p-1} \]  
\[ T^+ + H_2^+NR\beta_{p-1} \rightleftharpoons T^+H_2^+NR\beta_{p-1} \]

where \( T^+ \) is the dipolar form of tryptophan, \( T^- \) is the anionic form of tryptophan, \( H_2NR\beta_p \) is the albumin molecule without an associated proton at the binding site, \( H_2^+NR\beta_{p-1} \) is the albumin molecule with an associated proton at the binding site, and \( Z_p \), as before, the net charge on the protein which is always negative in these binding studies. For simplicity it is assumed that \( k_1 \), the intrinsic binding constant (uncorrected for possible effects of chloride binding), is the same for the association of the molecular forms in Equations 4 and 5. That the constants for these two equations probably do not differ greatly is seen in the studies with the esters of tryptophan. Binding between the forms in Equation 4 is considered to predominate. The observed amount of binding between the forms in Equation 5 is small because of the magnitude of the electrostatic repulsion between the 2 like-charged molecules. The association of the forms in Equation 6 is considered unimportant since only small
Binding of L-tryptophan to serum albumin after modification of albumin or addition of inhibitors

<table>
<thead>
<tr>
<th>Preparations</th>
<th>pH</th>
<th>Buffer</th>
<th>$k \times 10^{-4}$</th>
<th>$n_1$</th>
<th>$k_{max} \times 10^{-4}$</th>
<th>$n_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Addition of oleate to bovine serum albumin, Fraction V</td>
<td>7.95</td>
<td>Tris*</td>
<td>5.1</td>
<td>0.95</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No oleate added</td>
<td>7.95</td>
<td>Tris</td>
<td>5.1</td>
<td>0.95</td>
<td></td>
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<tr>
<td>0.5 M Oleate: M albumin</td>
<td>7.95</td>
<td>Tris</td>
<td>4.4</td>
<td>0.87</td>
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<td>1.0 M Oleate: M albumin</td>
<td>7.95</td>
<td>Tris</td>
<td>3.9</td>
<td>0.80</td>
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<tr>
<td>2.0 M Oleate: M albumin</td>
<td>7.95</td>
<td>Tris</td>
<td>2.3</td>
<td>0.75</td>
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<td>(b) Acetylated albumin</td>
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<td>PO$_4$</td>
<td>5.5</td>
<td>0.9</td>
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<td>0.55</td>
<td>0.15</td>
<td>&gt;3</td>
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<tr>
<td>Preparation 135CI</td>
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<td>0.3</td>
<td>0.15</td>
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<td>PO$_4$</td>
<td>(0)</td>
<td>(0)</td>
<td>0.15</td>
<td>&gt;3</td>
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<td>(c) Guanidinated albumin</td>
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<td>PO$_4$</td>
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<td>0.96</td>
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<td>10 M decanol: M albumin</td>
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<td>0.67</td>
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<td>(0)</td>
<td>0.05</td>
<td>&gt;3</td>
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</tbody>
</table>

* Tris (buffer) represents 2-amino-2-hydroxymethyl-1,3-propanediol.

concentrations of these two forms exist simultaneously. The forms in Equation 7 are assumed not to bind, since these forms predominate at low pH at which binding does not occur.

Considering that association occurs only between the forms in Equations 4 and 5, a theoretical curve for the observed binding can be calculated from the following relationships:

$$h' = \frac{(T^2 + T)^{-1}}{T^2 + T^{-1}} = \frac{(H_2NR^2+) + (H_2NR\bar{R}^2+)}{2H_2NR^2+}$$

and:

$$pH = pK'_{prot} - \log \frac{1 - \alpha}{\alpha}$$

where $pK'_{prot}$ is the negative logarithm of the dissociation constant of the protionic ionizing group on the protein. With a $pK'$ of 10.66 used for the dissociation constant of tryptophan the results of the binding studies in 0.1 M NaCl were fitted excellently with values of 1.3 x 10$^{-8}$ for $k_1$ and 8.3 for $pK'_{prot}$ (see solid curve in Fig. 3). This $pK'$ value, after adjustment for electrostatic effects by the equation of Tanford et al. (19):

$$pK'_{prot} = pK''_{prot} - 0.868ZpsilonA$$

gives a $pK''_{prot}$ value of 7.38 at 2°. The imidazole groups and the N-terminal aspartyl group of albumin have $pK''$ values of this general magnitude. The $pK''$ values of the imidazole groups have been estimated from titration studies (19) to be 7.35 at 2° (corrected from 25° using van't Hoff's equation and a $AH$ of 7000 calories). The $pK''$ value of the N-terminal aspartyl group has not been measured directly, but it can be inferred from values obtained for $\alpha$-amino groups on peptides that it is of a probable magnitude of 7 to 9 (20, 21). Later experiments with modified albumins tend to support the participation of the aspartyl amino group at the binding site.

The pH dependence of binding and the magnitude of binding of tryptophan to bovine serum albumin were found to be similar to those aspects of binding to human albumin.

**Effect of Fatty Acids on Binding of L-Tryptophan**

Long chain fatty acids are bound very tightly by albumin (22). The bovine mercaptalbumin used in the present study contained approximately 1 mole of fatty acid per mole of protein. This fact, together with the observation that the number of moles of tryptophan associated with albumin was not a whole number (° not 1, but less than 1), prompted an investigation as to whether fatty acids on the albumin were blocking additional binding sites of tryptophan.

Three separate preparations of bovine mercaptalbumin were treated by the method of Goodman (23) to remove the long chain fatty acids. It was found that this treatment had not significantly changed the number of sites at which tryptophan was bound. However, because of the unusual conditions to which albumin is subjected by this treatment, no detailed binding study was conducted to determine whether a small increase in tryptophan binding might have occurred which could have increased the number of sites from 0.94 to 1.

To assess further the effect of fatty acids on tryptophan-binding a study of tryptophan-binding in the presence of added oleate was made. The results of these studies are reported in Table III(a). For each mole of fatty acid added per mole of albumin the number of sites binding tryptophan decreased by approximately 0.10. Fatty acids do, therefore, interfere with tryptophan-binding to a minor extent, and their presence in the albumin samples could cause such a decrease in the values of $n$ as has been observed in the previous studies (0.94 instead of 1.0).

**Binding of L-Tryptophan to Modified Albumins**

**Acetylated Albumin**—The results of tryptophan-binding studies with the acetylated preparations described in Table I are shown in Table III(b). The decrease in the intercept on the y axis correlated reasonably well with the extent of acetylation of the N-terminal group of the albumin and indicated that when, for example, 50 per cent of the albumin molecules had been acetylated, only the remaining 50 per cent would bind tryptophan (see Table IV). This type of correlation suggested that acetylation of the N-terminal group blocked binding of tryptophan.

Acetylation of an $\epsilon$-amino group could explain such blocking if one $\epsilon$-amino group were acetylated preferentially at about the same rate as the $\alpha$-amino group. In view of the binding experi-
ments on guanidinated albumin, the participation of an ε-amino group in this manner does not appear likely. If this reduction in binding were attributable to a general effect of acetylation on the albumin molecule, such as might arise from decreased molecular charge, a change in the magnitude of the association constant rather than in the number of binding sites would be expected. This was not supported by the data. Furthermore, with an average of only five groups acetylated on the albumin, as in preparation 135BI, the changes in electrostatic effects would be small. It thus seems most likely that acetylation blocked a particular group in the vicinity of the site. The possibility of having acetylated groups other than the α-amino and ε-amino in these preparations is considered remote (24, 25).

Guanidinated Albumin—Table III(c) summarizes studies of the binding of L-tryptophan to guanidinated albumins prepared as described in Table I. The α-amino group was found to be guanidinated only after very extensive reaction, and then to the extent of only 10 to 20 per cent. The ε-amino groups, on the other hand, were guanidinated to various degrees. Preparations 5AII and 5CHI, 65 and 70 per cent guanidinated, respectively, showed no large alteration in binding affinity for L-tryptophan; only a slight change occurred in the ϱ intercept. Preparations 135EI and 31AII with the ε-amino-groups guanidinated 95 and 85 per cent, respectively, showed a significant decrease in binding of L-tryptophan. It should be noticed, particularly in one of these preparations (31AII), that ϱ, the number of sites on the albumin molecule, was not diminished; but that it was rather the association constant, k′, which had been reduced. This indicated that the guanidinated protein continued to bind tryptophan but with decreased affinity. This is in contrast to acetylated albumin in which the binding site is noncompetitively blocked.

Several explanations can be given for the effects of guanidination on binding. The decrease in binding after 43 groups, or 70 per cent of the ε-amino groups, had been guanidinated, suggested that a critical change was taking place in the later stages of the reaction. This could mean that a single group at the site became susceptible to guanidination after about 43 of the ε-amino groups had reacted; such a guanidinated group could partially block the site and lead to such a reduction in binding as was observed. Guanidination might also produce a general disorientation of the protein molecule resulting in a reduction in the affinity of tryptophan for the site. The present evidence is not sufficient to distinguish whether it is an individual group near the site of binding or an over-all molecular change that caused the decrease in binding affinity.

In a second study the pH dependence of tryptophan-binding to guanidinated albumin was measured (Preparation 31AII, Table III(c)). In this instance, as with unmodified albumin, the binding affinity for tryptophan was observed to go through a maximum near pH 9. Guanidination had evidently not changed the pK of the group on the protein which had caused binding to decrease below pH 9. Above pH 9 the decreased binding was as expected if the electrostatic repulsive forces between the albumin molecule and the anionic form of the tryptophan were the major cause for the decrease, as was previously suggested for unmodified protein.

Acetylated-Guanidinated Albumin—Guanidinated albumin preparations 5AII and 5CHI, in which the ε-amino groups were 65 and 70 per cent covered, respectively, and the α-amino group almost completely free, and which bound tryptophan with about the same affinity as the unmodified albumin, were partially acetylated (Table I). The binding of tryptophan was completely suppressed by this treatment. Amino acid analyses indicated that the α-amino group was more than 60 per cent acetylated, whereas, only about two and four of the remaining free ε-amino groups were acetylated. Acetylation would not occur at groups which had been guanidinated. These results support the requirement of an unreacted α-amino group on the protein for binding to occur.

Nonbinding of L-Tryptophan

It was surprising to find that albumin had essentially no affinity for the D-isomer of tryptophan (Figs. 4a and 4b). The small amount of binding observed was of the same order of magnitude as had been found for the binding of L-tryptophan to its secondary sites. This is believed to be the only instance in which stereospecificity of such a high degree has been found to occur in binding of compounds to albumin. Stereospecificity of a much smaller degree was observed by Karush (26) in studies of the D- and L-isomers of the anionic dye, 4-(4'-dimethylaminoazo)phenyl-N-benzylationanacetate.

![FIG. 4. (a) Binding of L-tryptophan to bovine mercaptalbumin, 0.05 M Tris buffer, pH 7.75, 0.1 M NaCl. ○, L-Tryptophan; △, L-tryptophan. (b) Binding of D-tryptophan to bovine mercaptalbumin, 0.05 M phosphate, pH 7.25. X, D-Tryptophan; △, L-tryptophan.](http://www.jbc.org/)

### Table IV

<table>
<thead>
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<th>Preparations</th>
<th>ϱ Intercept*</th>
<th>Percentage of groups free</th>
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<td>α-Amino</td>
<td>ε-Amino</td>
</tr>
<tr>
<td>Unmodified</td>
<td>0.9</td>
<td>100</td>
</tr>
<tr>
<td>135BI</td>
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<td>50</td>
</tr>
<tr>
<td>135CI</td>
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<td>20</td>
</tr>
<tr>
<td>135DI</td>
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<td>0</td>
</tr>
</tbody>
</table>

* The intercepts in this table were obtained after subtracting the approximate amount of tryptophan bound at secondary sites (see Table III(b)).

<table>
<thead>
<tr>
<th>Preparations</th>
<th>ϱ Intercept*</th>
<th>Percentage of groups free</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<tr>
<td>135BI</td>
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<tr>
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<td>20</td>
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<tr>
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</table>
Studies of binding with tryptophan analogues were undertaken to obtain information on the binding site. Analogues, modified either at the α-carbon or at the indole ring, were used to study the effect of the various groups which could be involved in binding. To assess the binding of these compounds, studies were first undertaken with the analogues alone, followed by competitive studies with L-tryptophan to ascertain that the same site was involved in the binding of both.

N-Acetyl-L-Tryptophan—N-Acetyl-DL-tryptophan had previously been reported to bind to serum albumin (27, 28), although a detailed investigation of its binding was not published. A study of the binding of N-acetyl-L-tryptophan to albumin as a function of pH was thus undertaken (Fig. 5). At pH 7.85 and higher the number of sites and magnitude of the binding constants are essentially constant. Below this pH, binding decreases, being about one-half its maximal value at pH 6.35. A decrease in binding in the pH region of 7.6 to 6.4 was also observed by Klots et al. (29), and by Karush (30) in studies with methyl orange and related dyes. The reduced binding at this lower pH does not appear to be directly attributable to any ionizable group on the protein (19). A large increase in chloride-binding at the lower pH could explain this anomaly.

N-Acetyl-L-tryptophan was bound to at least two sets of sites. A calculated curve with \( k_1 = 1.9 \times 10^5 \); \( n_1 = 1 \) and \( k_{2n} = 10^4 \) (\( n_2 \) taken as 25) fitted the data at a pH of 7.85 and above. In this case, as with the study of L-tryptophan, the constant \( k_1 \) and site \( n_1 \) (treated as an integer) were essentially fixed. It was found, however, that a better fit would have been obtained with the experimental points of acetyl-tryptophan if \( n_1 \) had been considered slightly less than 1. A value of less than 1 has also been suggested on the basis of the binding studies with \( n \)-tryptophan. In a desire to maintain stoichiometry, however, a value of less than 1 was not used in determinations of \( k \) values.

Competitive binding studies with N-acetyl-L-tryptophan and L-tryptophan were also undertaken. Fig. 6 clearly shows the reduction in binding of acetyl-tryptophan in the presence of L-tryptophan. The binding of L-tryptophan was similarly reduced in presence of acetyl-L-tryptophan. The finding that \( n \)-tryptophan and N-acetyl-\( n \)-tryptophan were bound at the same position on the protein indicated that a charged α-amino group on the tryptophan was not necessary for binding. Indeed binding with a higher intrinsic affinity occurred with acetyl-tryptophan, which was uncharged. Also the positively charged group at the protein binding site, which had prevented tryptophan from binding at the lower pH values, did not prevent acetyl-tryptophan from binding under similar conditions.

Esters of Tryptophan—In Fig. 7, a binding study of L-tryptophan ethyl ester as a function of pH is plotted with no electrostatic correction applied. If an electrostatic correction is included for the charged forms, using 8.26 as the pK for the ester (determined by titration) and assuming that the charged and uncharged species both were bound with association constants of the same magnitude, i.e. \( [(\bar{P}/A) \times \text{fraction of ester uncharged} + (\bar{P}/A) \times \text{fraction of ester charged}] \) plotted versus \( \bar{P} \)—the pH dependence of binding to the primary site is found to be similar to that of L-tryptophan except that the binding above pH 8.88 did not decrease but remained constant. Appreciable binding was also observed at secondary sites; however, in no instance could a calculated plot be fitted to the data unless it was assumed that one binding site had a considerably higher affinity than the others. The constants calculated for the bind-
ing at the higher pH levels, i.e. above pH 8.88, were $k_1 = 1.0 \times 10^4$, $n_1 = 1$, and $k_2 = 2.4 \times 10^4$ ($n_2$ taken as 25).

In the pH region studied there were two forms of the ester and two forms of the protein available to associate in complex formation at site 1. The pH of the ester amino group was found to be 8.29 whereas a pH of 8.3 had previously been calculated for the protein group from studies with L-tryptophan. The equilibrium equations of these forms are analogous to the equations 4, 5, and 7 except that the negative charge of tryptophan has been removed. The formation of a complex between the ester with a charge on the a-amino group and the protein with an associated proton at the binding site (equation 7) was not indicated in this study, nor had it been indicated in the binding studies with L-tryptophan. It was not possible to determine from the data values for the binding constants of the other forms. However, from Fig. 7, in considering binding to the first site, it can be seen that the slopes of the curves at pH 7.53 and 7.93 are not very different from that at pH 9.56. If the uncharged forms alone were bound, a large increase in slope should have occurred at the higher pH where the ester and group at the protein site are approximately 95 per cent uncharged. Since this was not observed, the implication was that these two forms were not the only forms associated. On the other hand some association between the uncharged species was necessary, or else a pronounced decrease in binding would have been found at the higher pH at which equilibrium was not favorable for association of the forms represented by equations 4 and 6. It can also be inferred from these data that the charged form of the ester must bind to some extent to account for the steepness of the slope at pH 7.53 and 7.93. (The electrostatic attraction between the positively charged ester and the negatively charged albumin molecules at pH 7.53 was of such a magnitude that $k'$ was 5 times greater than $k$.) Therefore, although individual constants for the third equilibria could not be computed with exactness it can be seen that the ester associates with the protein to some extent in both the charged and uncharged forms.

Binding studies of L-tryptophan methyl ester and D-tryptophan methyl ester were undertaken at pH 8.88 (Figs. 8 and 9, upper curve). After applying an electrostatic correction to the charged forms in the same manner as with the ethyl ester (using pH of the methyl esters as 8.26) the constants become for the methyl-L-ester: $k_1 = 3.1 \times 10^4$, $n_1 = 1$, and $k_2 = 1.6 \times 10^4$ ($n_2$ taken as 25) for the methyl-D-ester: $k_1 = 3 \times 10^3$, $n_1 = 3$.

Competitive binding of the methyl and ethyl esters with L-tryptophan was studied at pH 8.88. The lower curves in Figs. 8 to 10 inclusive summarize graphically the results of these competitive binding studies. In the presence of L-tryptophan the binding of the two L-esters was markedly reduced. The binding of L-tryptophan was also reduced proportionally to the amount of ester added. L-Tryptophan methyl ester, which was not bound preferentially to one site, showed only a small amount of competition to the binding of L-tryptophan. Here again stereospecificity was demonstrated. It was of interest that the amount of competition shown with the D-ester could not be attributed entirely to effects at secondary binding sites of tryptophan, but that at least a small part of it appeared to occur at the primary site of tryptophan. This was indicated by the reduction in both tryptophan and ester binding when both compounds were equilibrated together with albumin. The dotted line in Fig. 9 is the calculated reduction in binding of
the $\delta$-ester, based on the assumption that tryptophan was competing for one site. The agreement with the observed reduction is good. It suggests, therefore, that one of the three sites at which $\delta$-tryptophan methyl ester binds is the same as the primary site for tryptophan. The significance of this will be discussed when the forces at the site are considered.

**Other Tryptophan Analogues**—Binding studies of tryptazan and some methylated analogues of tryptophan were also made. Several of these compounds were racemic, which made assessment of the magnitude of binding difficult; the $\delta$ form would be expected to bind only to a small extent whereas the $L$ form would contribute most to the observed binding. $\deltaL$-Tryptazan and $6\delta$-methyl $\deltaL$-tryptophan showed an appreciable affinity for one site. The affinity constant for $\deltaL$-tryptophan was about one-half that of $L$-tryptophan, which would indicate that the $L$ form was being bound with about the same affinity as $\delta$-tryptophan. The binding by the $6\delta$-methyl $\deltaL$-tryptophan compound, on the other hand, was of about the same magnitude as the binding by $L$-tryptophan. This showed that the $\delta$ isomer was binding much more strongly than $L$-tryptophan. $5\delta$-Methyl $\deltaL$-tryptophan and $\alpha\delta$-methyl $\deltaL$-tryptophan were bound much more weakly than were the other analogues.

A competitive binding study of these compounds with $L$-tryptophan was also undertaken. Both $6\delta$-methyl $\deltaL$-tryptophan and $6\delta$-tryptizan were found to compete with the binding of $L$-tryptophan. $\alpha\delta$-Methyl $\deltaL$-tryptophan, on the other hand, was not competitive. Unfortunately, $5\delta$-methyl $\deltaL$-tryptophan did not resolve from tryptophan in either of the two solvents used for chromatography, and it could not be determined by this experiment whether it was bound at the primary site of tryptophan. However, the small amount of binding which it exhibited suggested it would not compete to an appreciable extent with tryptophan.

Tryptamine and 5-hydroxytryptamine were found not to bind with serum albumin. The significance of this will be further discussed.

Guandilated $L$-tryptophan did not bind to albumin. Presumably the large planar guanidinium group could not be accommodated at the site. This analogue was of interest since it remains in the dipolar form at higher pH values than does tryptophan.

**Inhibitory Effects of Other Compounds on $L$-Tryptophan Binding**

Decanol in a ratio of 10 moles to 1 of albumin was found to reduce binding of tryptophan by about 30 per cent (Table III (d)). An experiment in which 24 moles of decanol were added per mole of albumin showed no tryptophan-binding. The affinity of decanol for albumin could not be determined from the data. However, an indication of the magnitude of the binding constant could be obtained from the experiment at pH 7.85 in which the value of $V$ was reduced to 0.67 without appreciable change in the slope of the plot of $V/A$ versus $E$. This decrease in the availability of the site suggested that the affinity of decanol for albumin was very high in comparison with that of tryptophan. It also indicated that decanol was bound at a large number of sites ($n > 10$). Albumin crystallized by addition of decanol (16) was found not to bind tryptophan.

The inhibitory effect of benzene was studied by saturating the albumin solution with benzene before addition of tryptophan. Under these circumstances the binding of tryptophan was largely suppressed. Some indication of a benzene-albumin interaction had previously been given by the observation that albumin crystallization was enhanced when benzene was added to the solution (16). Also, it had been shown by Karush (31) that toluene inhibited binding of dyes.

$L$-Phenylalanine was found to inhibit the binding of $L$-tryptophan to a slight extent at pH 9. The association constant estimated from competitive studies was approximately 100-fold less than that of $L$-tryptophan.

The binding of $L$-tryptophan to albumin was unchanged when cupric ions were added at a molar concentration equivalent to that of the albumin. This suggested that $L$-tryptophan and the cupric ions were not bound at the same site such as at the $N$-terminal group of the protein (32). The presence of ethylenediaminetetraacetate did not affect the binding of tryptophan, a finding which indicates that metal ions were probably not mediatory in the binding of tryptophan.

**Other Studies**

Albumin to which had been added 90 moles of OH- ion per mole of albumin ($pH \sim 13$), and which had mildly jelled on standing for 16 hours at $2^\circ$, did not bind tryptophan after the albumin had returned to solution upon dialysis against phosphate buffer, pH 8. A preparation to which 70 moles of OH- ion per mole of albumin had been added ($pH \sim 12.5$), and which had stood for 16 hours at $2^\circ$, bound tryptophan with approximately a $20$ per cent reduction in $n_i$.

Albumin dissolved in $4\times$ molar urea solution and passed through a deionizing resin column (2) did not change its binding to $L$-tryptophan after the urea had been removed by dialysis. Albumin reduced the enzymatic activity of tryptophan peroxidase to an extent which correlated with the degree of binding of tryptophan. Activity of the enzyme was largely restored by the addition of decanol to the reaction mixture, implying that the decanol had replaced tryptophan from its albumin complex.

**DISCUSSION**

It has been the intent of this study to determine the nature of the site and the forces involved in the binding of tryptophan to serum albumin. Previous workers have undertaken studies of a similar type with other compounds (33-35). Of great advantage to the present study was the fact that only one binding site needed to be considered. Furthermore, many analogues of tryptophan were available with which to study binding. It is proposed in this section to define the primary site in terms of all the evidence available.

**Forces of Binding**—The minimal free energies of formation of the several association complexes are given in Table V. The $-\Delta F^o$ values shown are calculated from association constants for the primary site of 0.1 m NaCl and are taken at the pH where the maximal value for the association constant was found after statistical and electrostatic corrections had been applied. The pH of the maximal values of the association constants for the $\delta$- and $L$-tryptophan methyl esters was assumed to be the same as that of the ethyl ester.

A comparison of the $-\Delta F^o$ values of the compounds in Table V is instructive in considering binding forces. In competitive studies $\delta$-tryptophan methyl ester was bound by approximately

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4 We are indebted to Dr. T. Tanaka and Dr. W. E. Knox for making these determinations.
2 kilocalories per mole less energy than L-tryptophan. Any association attributable to the presence of the indole ring would be the same for either enantiomorph, since this ring has freedom of rotation with respect to the α-carbon. Therefore, it is reasonable to postulate that competition between the isomers would occur at the indole-binding region of the protein site. The indole ring can contribute a large amount of energy of attachment by van der Waals’ forces. (The heat of vaporization, which is roughly considered to measure the energy available for interactions between hydrocarbon molecules (36), is 17.9 kilocalories for indole (37).) The studies with d-tryptophan methyl ester suggest then that somewhat less than 4.5 kilocalories of binding energy is supplied to the L-tryptophan-albumin complex by virtue of an attachment at the indole ring. The larger size of the indole ring in relation to the hydrocarbon groups of the other amino acids, and, therefore, the larger amount of van der Waals’ attraction it can supply (36), could explain why tryptophan binds to albumin and why none of the other amino acids is appreciably bound.

The binding of tryptophan was very slight and a binding constant was not determined. The $-\Delta F^\circ$ value was estimated to be less than 4.5 kilocalories. L-Tryptophan, which is similar to tryptophane but has an additional carboxyl group, is bound by an energy of 6.4 kilocalories or at least 2 kilocalories more than tryptamine. These data imply that the carboxyl group supplies a minimum of 2 kilocalories of binding energy to the association of L-tryptophan with albumin. The α-amino group appears to contribute little to the energy of association of L-tryptophan or its analogues. In addition to the low binding affinity of tryptamine, the insensitivity to binding of the α-amino group, whether it was charged or uncharged or whether it was modified as in acetyl-tryptophan, also implied no attachment of this group. The α-hydrogen group would not be expected to make a significant contribution to the energy of binding. Therefore, the forces of attachment are considered to be divided between the indole ring and the carboxyl group.

Steric Relations at Site—Fig. 11 is a photograph of a molecular model of tryptophan with certain positions lettered for easy reference. Site position a (Fig. 11) is the location of the α-amino group of tryptophan. Evidence that the N-terminal group of the protein is in the proximity of site a was obtained from two sources. In the first instance, the study of the pH dependence of tryptophan-binding indicated that an ionizable group on the protein with an association constant in the range of either the imidazole group or the N-terminal group was involved in the tryptophan-albumin complex. Since ionization of this group interfered with tryptophan-binding but not with that of acetyltryptophan, these two groups (i.e. the α-amino and the ionizable protein group) must be close enough to prevent binding when both are similarly charged. The second source of evidence that the N-terminal amino group on the protein is in juxtaposition to the α-amino group comes from acetylation studies. It was shown that an inverse relationship existed between the extent of acetylation of the N-terminal group and the amount of tryptophan bound. This decrease in binding with the acetylated preparations cannot be attributed to an acetylation of imidazole residues since acetylimidazole compounds are rapidly hydrolyzed (39, 40) even in neutral solution.

Some spatial tolerance at Site a is indicated; an acetyl group on the α-amino nitrogen of tryptophan can be accommodated without impairment of binding. However, when the α-amino group is replaced by the planar, rigid, and larger guanidinium group, binding is impeded. From this it can be concluded that the space requirements at this position are not unlimited. Furthermore, the distance between the α-amino and the N-terminal group on the protein must be sufficiently small to provide a minimal repulsive force equivalent to 2 kilocalories when both groups are positively charged. An approximation of the distance between the charges made from Coulomb’s law with the use of the equation of Waugh (36) indicates this to be 4.3 A (dielectric constant taken as 80).

Table V

<table>
<thead>
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<th>Compound</th>
<th>$k_1$</th>
<th>$-\Delta F^\circ$ (kcal/mole)</th>
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<tr>
<td>L-Tryptophan</td>
<td>$1.3 \times 10^8$</td>
<td>6.44</td>
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<tr>
<td>Acetyl-L-tryptophan</td>
<td>$1.9 \times 10^8$</td>
<td>6.65</td>
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<td>L-Tryptophan ethyl ester</td>
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<td>L-Tryptophan methyl ester</td>
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<td>5.65</td>
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<tr>
<td>d-Tryptophan methyl ester</td>
<td>$0.03 \times 10^8$</td>
<td>4.40</td>
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<tr>
<td>Tryptamine</td>
<td>$&lt;4.5$</td>
<td></td>
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</tbody>
</table>

Fig. 11. Molecular model of L-tryptophan. Letters relate to positions studied in binding complex with serum albumin.

We are indebted to Dr. B. W. Low for the loan of this model (38).
tion 5 on the indole ring (Fig. 11) emphasizes the importance of van der Waals' forces at the indole ring in the binding of tryptophan. The steric effect of the methyl group prevented an appreciable part of this ring system from fitting closely. Methylation of the indole ring at position 6, however, increased binding. A methyl group on this part of the ring therefore enhances short range van der Waals' interactions at the protein-binding site. Further evidence of the exacting specificity of the site for the indole ring was seen in the binding studies with L-phenylalanine. The latter differs from tryptophan only in having a phenyl ring instead of the indole ring. The very low competitive effect of phenylalanine on tryptophan-binding clearly indicates the inability of the phenyl residue to substitute for the indole residue.

Position d is at the α-hydrogen. The replacement of this hydrogen by a methyl group (α-methyl tryptophan) blocked binding, indicating that close steric relationships existed at this location. The sensitivity of this site to the introduction of even a small group suggested it was important to the stereospecificity of the binding complex.

For a small molecule to bind to a protein in a stereospecific manner, three reference points in the association are necessary. These may be either positions of attachment of the 2 interacting molecules or adapted spaces which permit the molecules to come together. For L-tryptophan the three positions of reference must be oriented from the α-carbon and, for isomer specificity, each must fit only one of the four groups from the α-carbon. From the evidence presented previously it can be assumed that the carboxyl group and the indole methylene group provide two exclusive attachments. The third reference point must then be the "close fitting" space adjacent to the α-hydrogen. Evidence has been presented that the α-amino group (the only other group available as a reference point) plays a secondary role in the tryptophan-albumin association, whereas the space requirement at the α-hydrogen is critical. Furthermore, since α-methyl tryptophan was not bound by albumin, it would seem very unlikely that the α-amino group, which is as large as the methyl group, could occupy site d. If the α-amino group cannot occupy this position, then the necessary stereospecifications have been met and the protein site can distinguish α- and L-isomers.

Position e is at the α-carbon. The sensitivity of this site to the introduction of a methyl group (α-methyl tryptophan) was not occupied by L-tryptophan. The evidence presented previously it can be assumed that the α-carbon-protein bond, and (c) a "close fit" to the protein at the α-hydrogen of the tryptophan which would not permit the α-amino group to occupy this position. Strong evidence for this "close fit" was obtained by the failure of α-methyl tryptophan to bind.

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REFERENCES


SUMMARY

The only plasma protein which binds L-tryptophan appreciably is serum albumin.

L-Tryptophan is bound to serum albumin predominantly at one site in a highly stereospecific manner. d-Tryptophan is bound with an affinity of about 100-fold less than L-tryptophan.

The pH dependence of binding of L-tryptophan and its analogues implies the presence of an ionizable group at the protein site which prevents binding of the small molecules when both this ionizable group and the α-amino group on the small molecule are similarly charged.

Binding studies with modified albumins (acylated and guanidinated) and the pH dependence of binding of L-tryptophan indicate that the N-terminal group of albumin is the ionizable group involved at the protein-binding site.

Binding studies with N-acetyl-L-tryptophan, L-tryptophan ethyl ester, L-tryptophan methyl ester, N-tryptophan methyl ester and tryptamine show that the carboxyl group of L-tryptophan is required for binding, whether it be charged as in L-tryptophan or uncharged as in the esters of tryptophan. The α-amino group of tryptophan has only a secondary role in binding.

Studies with tryptophan analogues have implicated the indole ring as a strong point of attachment at the binding site. Furthermore, since 6-methyl tryptophan is bound, whereas the 5-methyl analogue is not, it would appear that only certain areas of the indole ring comprise this point of attachment.

Three points of reference are necessary for a compound to bind to a site in a stereospecific manner. The specificity requirements for the tryptophan-albumin binding site are satisfied by virtue of (a) an indole-protein bond, (b) a tryptophan carboxyl-protein bond, and (c) a "close fit" to the protein at the α-hydrogen of the tryptophan which would not permit the α-amino group to occupy this position. Strong evidence for this "close fit" was obtained by the failure of α-methyl tryptophan to bind.
The Specific Binding of L-Tryptophan to Serum Albumin
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