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The binding of dyestuffs by proteins

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BOSTON UNIVERSITY
GRADUATE SCHOOL

Thesis

THE BINDING OF DYESTUFFS BY PROTEINS

by

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(S.B., Simmons College, 1919)

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requirements for the degree of

Master of Arts

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EFFICIENCY BOND

FRAG CONTENT

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THE BINDING OF DYESTUFFS BY PROTEINS

The experimental work conducted and reported here was not directed primarily at further elucidation of the theory of the binding of organic ions by proteins. The purpose was rather to demonstrate to what extent such binding of dyestuff ions by proteins might serve as a useful and reliable analytical tool in the study of proteolysis and of protein synthesis.

Attempts to produce proteins by reversal of enzymatic proteolysis in vitro have been only moderately successful. By using amino-acid mixtures in high concentration it has been possible to produce protein-like polypeptides-----"Plasteins"-----in the presence of proteolytic enzymes, or even in the absence of such enzymes when certain aromatic compounds are present (Wasteneys and Borsook).

Maver, Johnson, and Voegtlin (19) claimed to have demonstrated reverse proteolysis in oxygenated preparations of ground rabbit tissue, both normal and tumorous. Their procedure was to measure protein and non-protein nitrogen in successive samples.

Benjamin Carrol (5) has demonstrated that action of a proteolytic enzyme upon bovine serum albumin causes it to lose its combining capacity for anionic dyes. His experiments were carried out in phosphate buffer at pH 5.4 (ionic strength not stated: the substrate was bovine albumin Armour) 0.2 percent; the enzyme was pepsin (Armour crystallized from porcine mucosa) 0.1 percent; the dye was Orange I, 2×10^{-5} M. Extinctions at 475 millimicrons were maximum in solution of dye alone, diminished

in solution of dye plus albumin, increasing with reaction with pepsin at 37 ± 2 C.

The work of Carrol has made it clear that, at least under his experimental conditions, dyestuff absorption by protein can be used as a measure of proteolysis. The work to be described is intended to explore the possibilities and limitations of this method in the study of protein synthesis.

EXPERIMENTAL

Orange I has a molecular weight of 350.16; a solution 2×10^{-3} M. was prepared by dissolving 0.700 grams in one liter of water. Absorption curves were made at pH 6 and pH 8 (see Fig. I). Note that at the wavelength used by Carroll---475 millimicrons---there is no significant difference in the absorption at the two pH values.

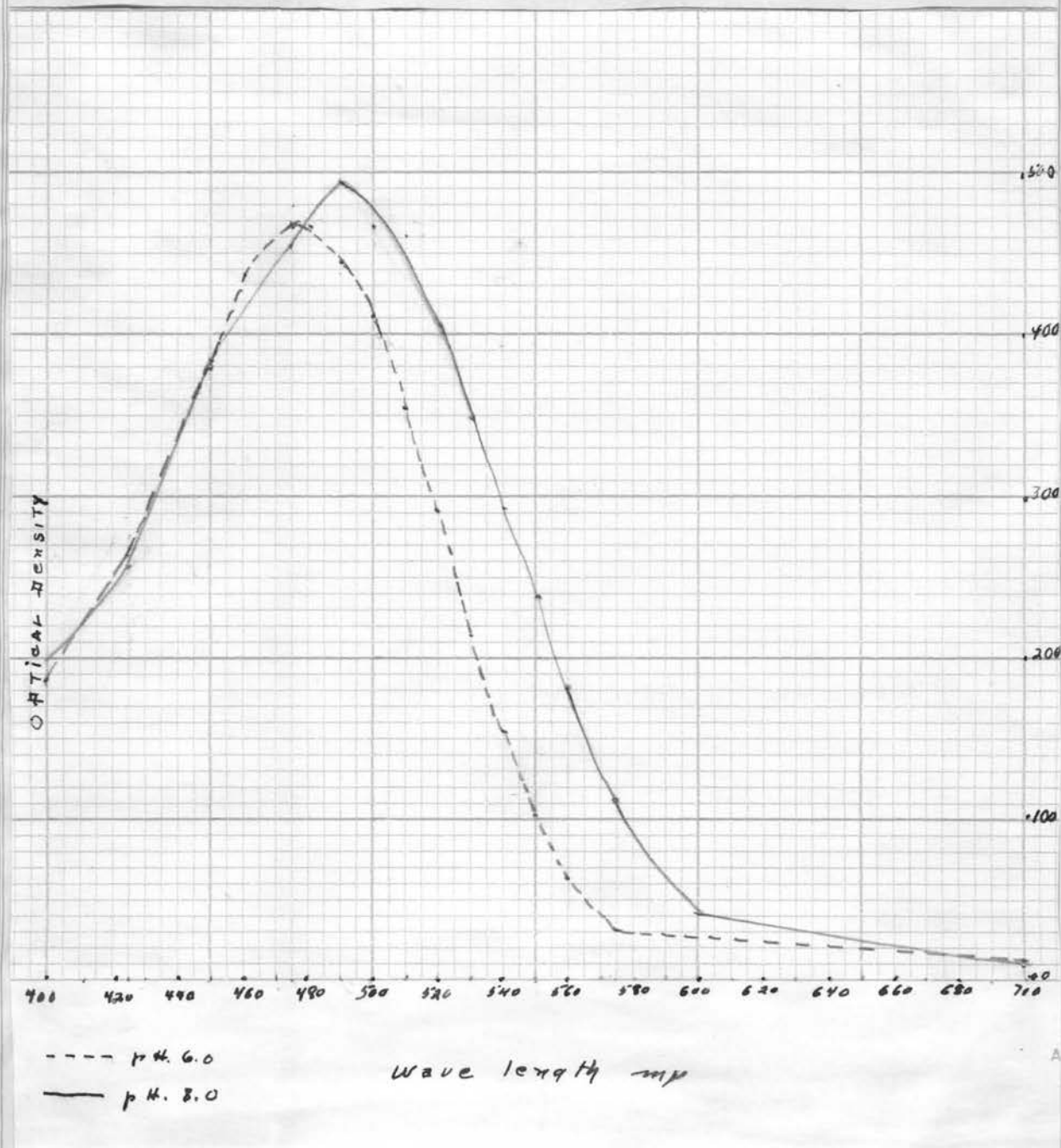
Attempts were made to sterilize mixtures of Orange I and protein by filtration. This was unsuccessful since Seitz, Berkefeld, and Hormann filters removed the dye. The dye was, however, found to be stable to heat sterilization in the autoclave. This question of sterilization arose since in future work in enzymatic synthesis of proteins it is proposed to work with sterile preparations to avoid possible errors by accumulation of microbial protein.

Attempts were also made to apply the procedure suggested by Carrol to the digestion of egg albumin by pepsin and by trypsin. No change in extinction was noted with the progress of hydrolysis. It is apparent that egg albumin is an unsatisfactory substrate for proteolysis where

Figure I

2b

Absorption Curves of Orange I (2×10^{-5} M) in Phosphate Buffer M/30 at
pH 6 and pH 8



dye-binding is to be used as the indicator. It was then decided to make a direct test of the binding capacities of Orange I by varying known amounts of protein.

For this purpose we used serial dilution made of human blood plasma in phosphate buffer M/15 and pH 7.4; one ml. of each dilution was measured into an Evelyn tube, and 10 ml. of Orange I solution, 2×10^{-5} M. in the same pH 7.4 buffer was added. These were read in the Evelyn photoelectric colorimeter at 470 μ with a water blank set at 100.

Table I

Binding of Orange I Dye by Varying Concentrations of Protein (Human Plasma)

<u>Final Percent</u>	<u>Percent Protein</u>	<u>Optical Density (2 - log G)</u>
0.55	6.0 added to 10 ml.	0.475
0.36	4.0	0.459
0.25	2.7	0.372
0.16	1.8	0.342
0.11	1.2	0.342
0.07	0.8	0.337
0.05	0.53	0.332
0.03	0.35	0.337
0.02	0.24	0.357
	Blank with dye	0.420

Inspection of Table I shows that in the lower concentration some binding of dyestuff occurs, but that in higher concentrations - concentrations refer to protein - there is an actual increase in optical density over that of the blank. This seemed to be the result of light absorption by the somewhat colored solution of the protein itself, which became insignificant at higher dilutions. Protein binding indicated between 0.5%-2.7%.

The decreased light absorption of solutions of Orange I in the presence of serum proteins was further demonstrated by the use of the Pulfrich Stufenphotometer. In one light path was placed a one-centimeter cuvette with phosphate buffer, pH 6.2, and a three-centimeter cuvette with $5 \times 10^{-7} M$ Orange I in horse serum diluted 3:100 with the same buffer. In the other light path was placed a one-centimeter cuvette with Orange I, $1.5 \times 10^{-6} M$ in the same buffer, and a three-centimeter cuvette with horse serum diluted 3:100 with the same buffer. In the absence of interaction between the dye and the protein, the transmissions of these solutions should have been identical. By actual measurement: (using filter S-47)

With the side containing the mixed dye and protein set at 50 percent transmission, the balancing readings of the other side were:

60.7	
60.8	
59.6	
60.8	Average: 60.5 percent

With the side containing the separated dye and protein, set at 100 percent transmission, the balancing readings of the other side were:

83.4	
82.2	
83.2	
82.0	Average: 82.7 percent

These results again demonstrate that in the presence of protein the absorbance of Orange I solutions is diminished. It now remains to show the extent of this decrease in absorbance and its quantitative relationship, if any, to protein concentration.

To measure this effect quantitatively, it was found necessary to prepare blank protein solutions, i.e., without dye, and to read each

protein-dye mixture against the corresponding blank with identical protein concentration.

Table II

The Binding of Orange I Dye by Varying Concentrations of Protein
(Human Plasma)
(Dye Concentration 2×10^{-6} M; pH 7)

<u>Protein Concentration</u> in Grams per 100 ml.	<u>Optical Density</u> ($2 - \log G$)	<u>Decrement</u>
0	0.854	
0.360	0.770	0.084
0.324	0.722	0.048
0.288	0.699	0.023
0.252	0.688	0.011
0.216	0.684	0.004
0.180	0.648	0.036
0.144	0.678	-0.030
0.108	0.638	0.040

These results seem to indicate that a proportionality exists between protein concentration and decrement in optical density (binding) at 470 m μ . between 0.10% and 0.30% protein. It now remains to be seen if this is specific for protein.

Solutions of sodium hippurate in concentrations between 1.0 and 0.1 M were taken in 10 ml. volume and 2 ml. of Orange I, 10^{-5} M added. A decrement in optical density was observed with increasing concentrations of hippurate. A similar decrement was observed with solutions of glycine. The results with these simpler compounds were less per unit change in concentration, but cannot be neglected. See pg. 10, Tables VI and VII.

Our observation on the binding of Orange I by human plasma closely resembled results obtained by Rawson (24). Using a dye concentration

M. 2×10^{-5} with gradual increases of plasma concentration from 0 to 3.0%. The dye showed an initial decrease in optical density to a minimum value which was interpreted as the point at which the maximum number of dye molecules were bound per molecule of albumin. With increase in plasma concentration the optical density increased. The composition of the dye protein complex changes from a high ratio to a low ratio of dye molecules per protein molecule during this increase in density.

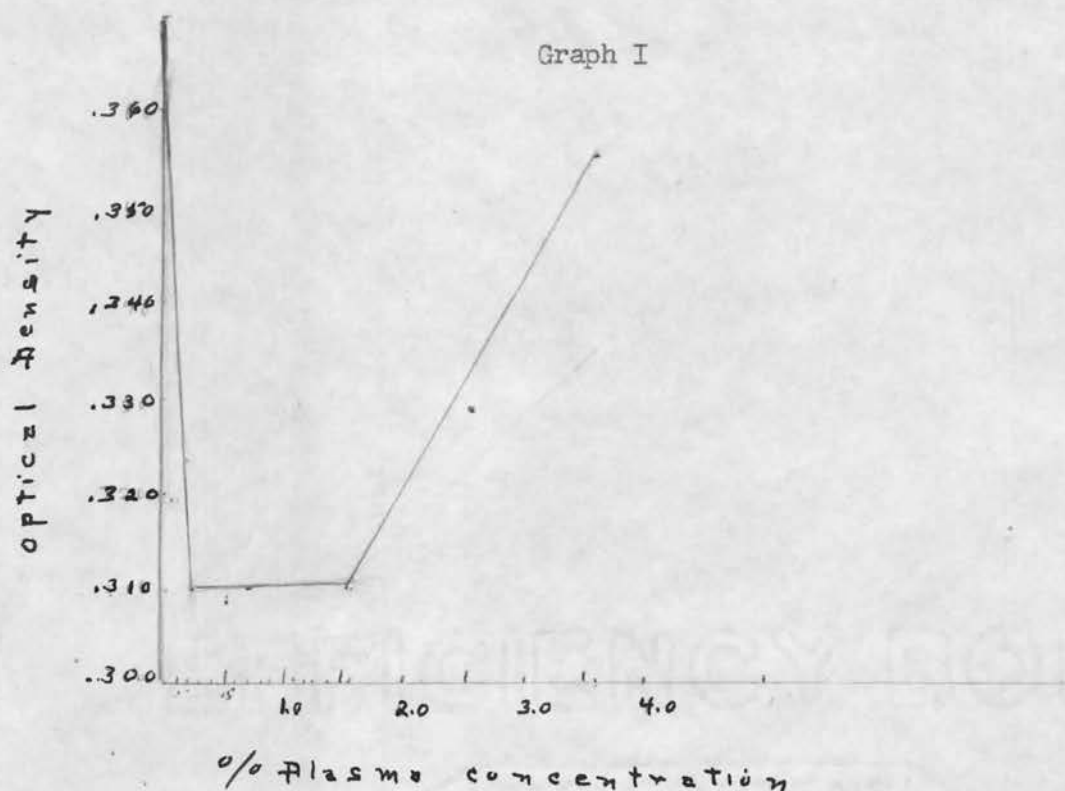
We investigated the binding of the dye Orange I by human blood plasma. Serial dilutions of plasma in phosphate buffer M/15 at pH 7.6 were made. One ml. amounts of each dilution were measured into Evelyn tubes containing 10 ml. of Orange I, concentration 2×10^{-5} M in the same buffer. These solutions were read in the Evelyn Photoelectric Colorimeter at 470 m μ . Control tubes of protein dilutions containing no dye were read at the same time.

Table III

The Binding of Orange I Dye by Varying Concentrations of Protein (Human Plasma)

<u>Protein Percent</u>	<u>Optical Density</u>
0.36	0.354
0.25	0.328
0.16	0.310
0.11	0.332
0.07	0.310
0.05	0.308
0.03	0.310
0.02	0.324
0.01	0.319

Variations in optical density proportional to the protein concentration in this investigation lie between 0.11% - 0.36% protein.



We studied the binding effect of Grublers neutral vital red by horse serum. Gregerson had shown a progressive increase in binding of this dye in proportion to the plasma concentration. (9)

Serial dilutions of horse serum were prepared in phosphate buffer solution M/15 at pH 7.0. One ml. of each dilution was measured into Evelyn tubes containing 10 ml. amounts of Grublers vital red dye $\times 10^{-5}$ M in the same buffer. Control tubes containing the same buffered protein solutions without dye were read at the same time. Read on Evelyn photoelectric colorimeter at 490 mm.

Table IV

The Binding of Dye, Grüblers Vital Neutral
Red, by Varying Concentrations of Protein (Horse Serum)

<u>Protein Percent</u>	<u>Optical Density</u>
0.48	0.2328
0.38	0.2273
0.31	0.2147
0.24	0.2076
0.20	0.1805
0.16	0.1772
0.12	0.1888
0.10	0.1838
0.08	0.1723
0.06	0.1838
0.05	0.1855

The proportional effect on the optical density of the dye by protein appeared to be in the range 0.16% to 0.48% \pm .

The binding properties of egg albumin for the dye Orange I was then investigated by us. Serial dilutions of egg albumin were prepared in phosphate buffer M/15 at pH 7.6. One ml. amounts of each dilution measured into Evelyn tubes containing 10 ml. amounts of Orange I (2×10^{-5} M) in the same buffer. Control tubes containing the same amounts of protein in the phosphate buffer but no dye were read on Evelyn at 470 mm. the same time and corrections made.

Table V

The Binding of Dye, Orange I, by Varying Concentrations of Protein (Egg Albumin)

<u>Protein Percent</u>	<u>Optical Density (corrected)</u>
0.67	0.492
0.44	0.492
0.27	0.508
0.18	0.501
0.12	0.499
0.08	0.470
0.05	0.486
0.04	0.484

Between 0.08% and 0.27% protein concentrations very slight changes in optical density were observed. These results show that egg albumin has extremely slight binding capacity for the dye Orange I at pH 7.6.

Klotz and Urquhart (17) had similar results when they investigated the combining capacities of a number of native crystalline proteins for the dye methyl orange at pH 6.8. The proteins studied by them were egg albumin, Beta lactoglobulin, insulin, lysozyme, ribonuclease, pepsin, trypsin, and chymotrypsin. Of these, they found that only Beta lactoglobulin showed appreciable binding. Egg albumin has been found, however, to bind small anions in solutions acid to its isoelectric point (19 and 28). It has been found that shifts in indicator equilibrium correspond to its binding affinity (18).

Table VI

The Effect Upon the Optical Density of the Dye Orange I Conc.
 2×10^{-5} M of Adding Sodium Hippurate

Sodium Hippurate in Phosphate Buffer at pH 6.2

<u>Sodium Hippurate Concentration</u>	<u>Optical Density</u>
1.0 M	0.2076
0.5 M	0.2147
0.2 M	0.2596
0.1 M	0.2756
0	0.301

Klotz (12), using individual amino acids in 0.2% concentration at pH 7.0, added to azosulfathiazole or to methyl orange, found no spectral change. They used amino acids arginine, histidine, lysine, glycine, tyrosine, and cysteine.

Our results follow:

Table VII

The Effect on Optical Density of Dye Orange I of Addition of Amino Acid, (Glycine)

Glycine M/1 in Phosphate Buffer M/15 pH 7.2
 Orange I (2×10^{-6} M)

<u>Glycine Concentration</u>	<u>Optical Density</u>
M/1	0.763
M/2	0.763
M/10	0.810
0	0.817

A decrease in optical density is produced by the addition of glycine to the dye Orange I.

We investigated the binding effects of human immune globulin for the dye Orange I. Serial dilutions of human serum immune globulin were prepared

in phosphate buffer solution M/15 at pH 7.0. One ml. of each dilution measured into Evelyn tubes containing 10 ml. amounts of Orange I concentration 2×10^{-5} M in the same buffer. Control tubes containing the same amounts of protein in buffer but no dye were read at the same time on Evelyn at 470 nm. (Squibbs gamma globulin, concentrated, was used).

Table VIII

Binding of the Dye Orange I by Immune Globulin Fraction
of Human Serum

<u>Protein Percent</u>	<u>Optical Density (corrected)</u>
0.27	0.464
0.18	0.459
0.12	0.466
0.08	0.469
0.05	0.474
0.035	0.469
0.015	0.465
0.01	0.436

There was no binding by human immune globulin of the dye Orange I. This result corresponds with the results obtained by Klotz and Urquhart who investigated the binding of methyl orange by bovine serum globulin. Klotz found no binding of the dye using 0.2% gamma globulin at several pH's: 5.8, 6.9, or 7.4. (12) (17)

In conclusion, it appears that the binding of Orange I will demonstrate significantly changes in serum albumin concentration in dilutions of 0.25 percent or less. Other proteins, as well as free and conjugated amino acids, produce less definite effects. On account of the apparent specificity for serum albumin, this method does not seem to offer a useful approach for the study of enzymatic protein synthesis in vitro.

III. Review of Investigations on Binding of Dyestuffs by Proteins

effects on the optical densities of the individual dyes. Initial sharp increases in optical densities of certain dyes were noted after addition of very small amounts of plasma; then a stability was observed over a long range. Brilliant Vital Red solutions increased in optical density with increase in plasma concentration. The dyes T-1824 and Brilliant Vital Red were found to conform to the Lambert Beer Law. They showed a strict proportionality between their optical densities and concentrations.

The electrophoretic patterns of normal human plasma containing the dye T-1824 have been studied by Rawson (24). At a concentration of 0.004 percent or less, the dye was found entirely in the albumin fraction. When excess of dye was used, it was bound by the alpha and beta globulins also, but not bound by gamma globulin. This investigator has studied the absorption curves of several dyes in human plasma, and also in the albumin and globulin fractions of plasma. The shape of the curve with 1.0% serum albumin was the same as that shown by the plasma. The changes in the absorption spectra were found to be mainly in the albumin fraction. The effect on the absorption spectra of varying the concentrations of electrophoretically pure albumin varied with each of several dyes niagra blue, niagra sky blue 6B and trypan blue.

Serum albumin has been found to have an unusual capacity to bind anions, above and below its isoelectric point. Klotz (11-18) and his associates have extensively investigated this binding capacity of solutions of crystalline bovine serum albumin. They made equilibrium dialysis studies on the binding by this protein (Armour) of two azosulfonic acids -

methyl orange and sulfathiazole (11). They employed cellophane sausage casing, which contained measured amounts of solution of crystalline albumin. These were immersed in measured amounts of the dye investigated until equilibrium conditions were reached, - these equilibrium conditions were found by experiment to be seventy-two hours at 5C, - the outer solution was then analyzed by spectrophotometer. The concentration of methyl orange used was $M 7.46 \times 10^{-5}$ in 0.1 M Phosphate Buffer at pH 5.68. Protein solutions were contained in the same buffer. The spectral shifts produced by the binding of azosulfathiazole by bovine serum albumin are of the same magnitude as those produced when this protein binds methyl orange. The maximum number of bound dye ions per protein molecule was found to be twenty-two, the same for each compound investigated. Klotz considers this reaction purely electrostatic, involving the reaction between the sulfonate groups of the dye, with the cationic groups on the protein.

The spectral properties of azosulfathiazole are typical of many dyes studied by Klotz. Its absorption of light was found to be directly proportional to its concentration up to 10^{-3} molar. This conformity to Beer's Law indicates that the dye is in a monomeric state in dilutions below 10^{-3} molar. Sodium chloride up to 0.5 molar has no significant effect on the dye spectrum. The absorption is independent of pH over the region 2 - 9. The presence of bovine serum albumin in concentrations from a few hundredths percent up to 0.2 percent in a dye solution of 1×10^{-5} M produces a progressive lowering of the absorption spectra. Increasing the protein concentration from 0.2% to 1.0% produces no further effect.

The binding capacity of bovine serum albumin for (12): Neoprontosil, Orange I, Orange II, and Methyl Orange has been investigated. The optical densities are increased by the addition of serum albumin to solutions of each dye. With each dye the shift in wave length differs, indicating that other groups beside sulfonate bindings are concerned. When sodium dodecyl sulfate is added to the dye protein mixture, no spectral shift is produced. Gelatine used instead of protein does not produce a spectral shift.

This binding which occurs between pH 5-9 indicates that the quaternary nitrogen of the epsilon amino groups of lysine or the guanidinium group of arginine are involved in this reaction. These groups would exist as positive charges in this range.

The addition of individual organic acids displaces the dye from the protein complex (13) and the extent of this reversal depends upon the concentration of the dye. The carboxyl and the sulfonate ions compete for reaction with the cationic group of the protein. This displacing ability of the organic acids decreases with increase in pH. Van der Waals forces would contribute more stability to the protein-dye-complex than to the protein organic ion complex. The anionic form only is effective in displacing the azo dye. The stability of the dye protein complex is determined by:

- (a) Electrostatic forces between the sulfonate ion, and the quaternary nitrogen of the amino groups, and
- (b) van der Waals interactions, particularly with the aromatic rings in the dye.

Van der Waals forces are considered to play a considerable part in these protein anion reactions which take place above the iso electric point of the protein. Pauling has described this effect as follows:

To every atom may be assigned a radius which describes its effective size with respect to inter-molecular reactions - a van der Waals radius. A molecule without a permanent dipole moment may have instantaneous dipole moments which produce an electric field. This will polarize, and attract any nearby molecule, and every atom in a neighboring molecule. It increases with the size of the molecule, and increases as the distance between atoms lessens. When two molecules have complementary structures the van der Waals force is large. (23) The magnitude depends upon the number of pairs of atoms which can be brought into contact.

When the presence of a competing ion, such as an organic acid displaces the dye from the protein dye complex, the spectrum is shifted toward that of the dye alone. Klotz has found by quantitative investigations that the binding affinity of the anion for the protein is correlated in general with the molecular weight, in so far as large variations are concerned. Within smaller variations of molecular weight, polar interactions with the aqueous solvent may play a part. The detergent sodium dodecyl sulfate, with a large anion, is bound strongly by serum albumin. It will completely displace methyl orange from its combination with this protein. Urea and urethane in concentrated solutions will displace the dye from the protein dye complex. This reaction is immediate and reversible.

Comparison of affinities of bovine serum albumin for compounds of equal molecular weight with a series of para and ortho isomers of amino benzene sulfonic acid, and hydrobenzoic acid show that the ortho

compounds are bound more securely than the para isomers. (17) Differences are attributed to strong interaction of the para compound with the aqueous solvent.

Quantitative observations on the interactions of bovine serum albumin at a pH above its isoelectric point with adenylic acid, adenine, and adenosine, (14) using the method of equilibrium dialysis gave evidence of binding by the albumin. The outer solutions were analyzed by the Beckman ultra violet spectrophotometer. The free energies of binding were found to be far below those of the dyes studied. The phosphate group, even with the same charge, increases the binding energy to a greater extent than does the ribose group of larger size, and increased van der Waals' effect.

It has been emphasized by Davis (7) that the antibacterial activity of the sulfonamides parallels their absorption by plasma and by micro-organisms. The binding capacity by bovine serum albumin of these sulfonamides has been determined by the equilibrium-dialysis technique. (15) The outer solutions were colorless; therefore, absorption of ultra violet light was used, and measurements made with a Beckman spectrophotometer. The number of molecules of bound dye per molecule of total protein in proportion to free dye were determined.

The free energy changes were determined for these reactions. The binding energies were increased with anion concentration, and decreasing pK_a . When two sulfonamides with equal anion concentration were used for binding serum albumin, the binding energy was greater for the larger anion due to van der Waals forces. Differences in the binding energies

of sulfapyridine and 2-sulfanilamido-5-chlorapyridine at pH 7.8 appear to be due to differences in ionization at this pH. Further studies (19) on the binding of serum albumin with dye show a decrease in the binding with an increase in pH. This decrease occurs in the pH range, where the quaternary nitrogens of the amino groups are losing their charge. The binding of serum albumin which the lysine amino groups had been selectively acetylated showed a marked drop in complex formation. This drop was not proportional to the decrease in the number of available lysine cationic groups, which may indicate that the guanidinium groups of arginine are also active in binding. A guanidinated serum albumin was then compared with its parent albumin. The binding measurements were identical.

Equilibrium reactions on the combining properties of the protein solutions, wool keratin, and egg albumin, with large anions were observed by Steinhardt. (28) He found that of thirty large anions studied, all would combine with these proteins, and that the tendency to combine increased with the molecular weight of the ion involved. Soluble complexes could be formed above the isoelectric point of the protein when reacting groups had the same charge. The effect was increased in the presence of salts. It was also observed that these large ions would catalyze the hydrolysis by dilute acids of peptide, and amide groups. This effect was increased with affinity of the anion for the protein. Kinetic studies of these reactions showed that these groups were dissociated in a less acid range than normally. Thiel and Schulz (29) found that casein binds methyl orange anion at pH near 3.0. It has been observed that the anions which bind strongly such as dodecyl sulfate, will become bound by a protein low

in the scale of binding affinities, as insulin and egg albumin.

The protein binding by large ions of detergents, such as sodium dodecyl sulfate, has been of considerable interest. Neurath and Putnam (22) found that equine serum albumin formed several complexes with dodecyl sulfate pH 6.7. When the concentration of the detergent was in the molecular ratio 55:1 there was no change in the viscosity of the complex. Doubling the ratio of detergent formed a combination with all of the cationic groups on the protein, denaturing it and disorganizing the protein, and the viscosity became greatly increased.

According to Mirsky and Pauling's (21) theory of protein structure, some of the side chain amino and carboxyl groups of the protein are free on the surface of the molecule. These react with dilute solutions. Other groups on the protein are involved in the formation of internal hydrogen bonds which maintain the identity of the protein. The penetration of the protein by a few large detergent ions will force the polypeptide chains apart and will finally result in the breaking down of the side chain linkages.

Lundgren and co-workers have investigated similar complexes between egg albumin and dodecyl benzene sulfate. (19) Duggan and Luck (8) more recently have employed dodecyl sulfate as a stabilizer of protein against denaturation. They tested several stabilizers on albumin mixtures in 6 M. urea and found dodecyl sulfate to be the most effective. Luck (1-3, 25) and his co-workers have found that sodium caprylate and related compounds have a considerable influence as stabilizers on thermal stability, on

electrophoretic mobility, and on the rate of papain digestion. They observed the effect on the rate of papain digestion of human serum albumin in 6 M.urea after the addition of sodium caprylate. Proteolytic enzymes, papain in particular, usually hydrolyze denatured protein more rapidly than native protein. Sodium caprylate in small amounts prevents denaturation. Sodium caprylate will also cause a marked decrease in viscosity of an albumin solution denatured by urea. It apparently causes the refolding of the partially unfolded molecule.

When a series of fatty acids were studied for effectiveness as stabilizers of serum albumin, it was found that stabilizing effects increased with the length of the hydrocarbon chain from acetate to caprylate. Sodium chloride though less effective as a stabilizer than acetate, adds considerably to albumin stability. (2)

Scatchard and co-workers (27) have found that the sodium salts of acetyl derivatives of certain amino acids phenylalanine, isoleucine, and tryptophan are excellent stabilizers. Acetyl tryptophan 0.04 M will protect serum albumin up to 60 C for several hours.

A concentrated serum albumin solution at pH 7.0 may, in the presence of 0.04 M sodium caprylate, be heated at 60 C for ten hours without denaturation. Evidently the large non-polar group attached to the anion makes an effective stabilizer. Albumin will bind saturated, and unsaturated fatty acids. It has a strong affinity for the oleate anion, and removes it quantitatively from solution. (6)

Recently it has been demonstrated by Klotz (16) and also by Scatchard (26) that bovine serum albumin will combine with the chloride ion. Karush (10) has shown that the chloride ion will compete with and inhibit the binding of dye by serum albumin. Buffer anions have also been shown to compete with dye anions for combination with serum albumin to a lesser extent. When different buffers were used at pH 5.7 and pH 5.0 in the binding of methyl orange by bovine serum albumin, a considerable difference was obtained in the value of the maximum number of anions bound by a single albumin molecule.

The presence of amino and carboxyl groups do not distinguish serum albumin; therefore, the unusual binding capacity of serum albumin must be due to other reactions than amino and carboxyl interactions within the molecule. Klotz (17) has advanced an explanation that a preferential reaction might be that of internal reaction between carboxyl, and hydroxyl groups. After this reacting has taken place the resulting excess ion will react with the amino group. Amino acid composition of several proteins were investigated. He converted them to a common unit weight and determined the number of carboxyl, hydroxyl, and amino groups.

Table IX

Amino Acid Composition and Binding Ability of Some Proteins (17)

<u>Protein</u>	$\Sigma(\text{COO}^-)$	$\Sigma(\text{OH})$	$\Sigma(\text{=NH}^+)$	$\frac{\Sigma(\text{=NH}^+)}{\Sigma(\text{COO}^-) + \Sigma(\text{OH})}$	$\frac{\Sigma(\text{=NH}^+)}{\Sigma(\text{COO}^-) - \Sigma(\text{OH})}$
Bovine Serum Albumin	133	128	115	0.55	29.
B Lactoglobulin	141	118	105	0.40	4.6
Egg Albumin	98	134	90	0.39	2.5
Casein	99	116	100	0.41	2.1
Insulin	63	150	72	0.34	0.83
Bovine Globulin	67	216	99	0.35	0.66

The $\frac{\sum (\equiv\text{NH}^+)}{\sum (\text{COO}^-) \sum (\text{OH})} = 29$ is extremely high for serum albumin and low for all the other native proteins. This may be termed a "binding index" or binding ability of a protein. The binding index is based on the distribution of the amino acids in a protein, and on the functional groups of these amino acids. Karush (10) investigated recently the binding ability of bovine serum albumin for an azo dye by the equilibrium dialysis technique. He found that at 25 C a three-fold increase in albumin concentration had no effect upon the binding with the dye used. He did have different binding values at 0 C, the temperature at which Klotz carried on his equilibrium investigations. Sodium chloride 0.10 M inhibited dye binding in this work. He found that his data filled the formula used by Scatchard.

$$r/c = \frac{n_1 + K_1}{1 + K_1c} + \frac{n_2 + K_2}{1 + K_2c}$$

where $n = n_1 + n_2$

$c =$ free dye

$n =$ no. of binding sites

$$r = \frac{\text{average no. of bound dye moles}}{1 \text{ mole albumin}}$$

He assumed 22 binding sites per mole of albumin which may be divided into a small group of 5 with a high binding constant, and a second group of 17 with small binding constant.

Karush emphasizes the configurational adaptability of the protein molecule which can take part in many diverse reactions. He believes that an aqueous solution of bovine serum albumin contains a large number of structural molecular species of equal energy in equilibrium with each other and characterized by a large configurational entropy.

Review of Experimental Work

The purpose of this investigation was primarily directed toward utilization of the method suggested by Carrol on the use of dyestuffs for determination of the activity of proteolytic enzymes. The method was based on the affinity of serum albumin for dye anions above, and below its isoelectric point. The optical density of an aqueous solution of a dye is increased by the addition of protein. This author has found that proteolytic enzymes denature serum albumin which results in loss of its binding capacity for dyes. Quantitative activity measurements were made by him of protein dye mixtures by estimating the concentration of protein in the presence of the enzyme as a function of the time. These observations were determined by spectro photometric analysis.

Our attempt to reproduce these results using human serum, and egg albumin were not successful. We then directed our efforts toward ascertaining the limits of accuracy, in determining the combining capacity of native protein for dyes. We have investigated the combining effects of human plasma for the Dye Orange I in dilute concentrations usually 2×10^{-5} M. Decreases in optical density of the dye proportional to the protein concentration were obtained.

	Protein Percent	Orange I Dye Conc.	pH
Table I	0.05 -- 0.27	2×10^{-5} M	7.4
Table II	0.18 -- 0.36	2×10^{-6} M	7.0
Table III	0.05 -- 0.36 (corrected)	2×10^{-5} M	7.6
		Grublers Vital Red	
Table IV	0.16 -- 0.48 (corrected)	$x 10^{-5}$ M	7.0

In Tables III and IV control solutions containing the same concentrations of protein in buffered phosphate without dye were read for correction purposes. When Grublers vital red was used, the limits of protein effect on the dye appear to be in a slightly higher range.

Our efforts to bind dye with egg albumin at pH 7.6 were unsuccessful. This conforms with results obtained by other investigators. Human immune globulin, (gamma globulin) evidenced no binding whatever when added to Orange I. We found that sodium hippurate decreased the optical density of the dye solution, and a decrease was also observed when glycine was added to the dye. These effects must be taken into consideration when the proteolytic activity of enzymes are considered in the presence of dye and protein.

Discussion

Investigations on the binding capacity of the serum proteins for dye anions and other small anions are comparatively recent. In 1932, the binding ability of blood serum was investigated by Bennhold. The ability of dye solutions to infuse gelatine used as an experimental method for studying the binding capacity of blood serum. It was observed that aqueous solutions of many dyes would infuse far into the gel, while serum containing dye would slightly infuse the gel. Evidence was obtained of protein dye binding. Fractionation of the serum by electro dialysis made it possible to observe bindings of serum fractions. Binding ability was found to be confined to the albumin fraction of the serum. Cataphoresis experiments on protein and dye showed the same effect.

Optical densities were determined on several dyes used in estimating plasma volume. It was observed that a variation existed in the binding effects for plasma by different dyes. Certain dyes did conform to the Lambert Beer Law. The optical densities of these dyes were directly proportional to their concentration in a constant solvent.

Electrophoretic patterns of serum containing dye showed that the albumin fraction only contained the dye unless excess dye was used. Optical density determinations of several dyes containing electrophoretically pure albumin showed variations in the combining affinities of protein with dye.

Quantitative studies have been obtained by Klotz on the binding of solutions of crystalline bovine serum albumin, by the method of equilibrium dialysis. The spectral properties of azosulfathiazole were found to be typical of many dyes studied. It was observed that the presence of bovine serum albumin in concentration from a few hundredths percent to 0.20 percent in a dye solution of 1×10^{-5} M, would produce a progressive lowering of the absorption spectra. Increasing the protein concentration from 0.2% to 1.0% produced no further effect. Our observations have indicated that increasing the protein concentration over 0.25% gave irregular results on the absorption spectra of Orange I concentration 2×10^{-5} M.

The forces contributing to the stability of the protein dye complex have been considered, the forces of electrostatic attraction, and van der Waals forces. Electrostatic effects are increased by ionization, and decreased by increasing pH due to loss of charge by the quaternary nitrogens of the amino groups and the guanidinium groups of arginine.

Van der Waals forces are increased by complementary structure, and by molecular weight increase. The complex formation of serum albumin with large anions has been considered. The complex apparently owes its stability to van der Waals forces. The competitive action of the organic acid anions, and the chloride ion for combination with albumin in the protein dye complex has been established. The stabilizing effects of small amounts of organic acid anions on albumin have been shown to increase in proportion to the length of the hydrocarbon chain from acetate to caprylate. Finally theoretical explanations concerning the binding capacity of serum albumin have been presented.

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