pric ions in albumin solutions (Fig. 3).[11] As has been pointed out by Borsook and Thimann,[10] in their work on amino acid complexes of copper, the absorption peak remains in the near infrared in complexes bound through the carboxylate substituent and moves toward shorter wave lengths when Cu–N bonds are formed. The absorption peak of cupric ions in the presence of protein is substantially in the same region as in copper chloride or copper sulfate solutions.

Despite these indications of the importance of the carboxyl group in binding the cation, there is no agreement between the maximum number of bound copper ions (16) and the number of aspartyl (56) and glutamyl (80) residues in bovine serum albumin.[12] A similar lack of correspondence with the content of basic amino acids has been found in investigations of the binding of anions by bovine albumin.[5] It seems apparent once again that the primary focus of binding on the protein, the carboxyl group in Cu^{++} binding, must be in suitable juxtaposition with other substituents or residues before a stable complex can be formed.

Acknowledgment.—These investigations were carried out with the aid of a grant from the Office of Naval Research. The authors are indebted also to Professor George Scatchard of the Massachusetts Institute of Technology for several suggestions on improving the treatment of the data.

Summary

1. Measurements have been made of the binding of cupric ions by bovine serum albumin at a *p*H of 4.8 and at temperatures of 0° and 25°, respectively.

2. The results obtained have been correlated in terms of equations derived from statistical and electrostatic considerations. Free energies, entropies and enthalpies have been calculated for the multiple equilibria involved.

3. The decrease in binding with decrease in *p*H, as well as the character of the absorption spectrum of the copper–albumin complex, indicates the importance of carboxyl groups on the protein in the bond with the cation.

(11) We are indebted to Miss Jean Urquhart for recording the spectrum of the copper-protein complex.

(12) E. Brand, *Annals N. Y. Acad. Science*, **47**, 187 (1946). Only 37 glutamic acid residues are listed as free.

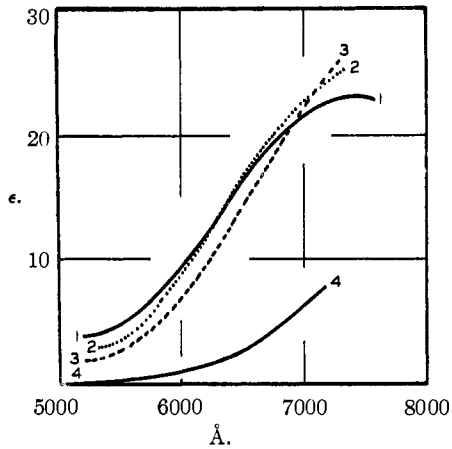


Fig. 3.—Absorption spectra of some copper complexes: 1, cupric ions with 2% bovine serum albumin at *p*H 4.23; 2, copper acetate in alcohol[10]; 3, cupric ions with glycine[10]; 4, cupric chloride *p*H 4.66.

EVANSTON, ILLINOIS RECEIVED OCTOBER 24, 1947

[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF NORTHWESTERN UNIVERSITY]

The Binding of Some Sulfonamides by Bovine Serum Albumin

BY IRVING M. KLOTZ AND F. MARIAN WALKER

Numerous studies have been made of the binding of sulfonamides by plasma.[1-3] From a pharmacological point of view it has been emphasized, particularly by Davis,[1] that the distribution of sulfonamides in various body fluids is strongly dependent on the extent of their binding by proteins. Similarly in connection with their chemotherapeutic properties, it has been pointed out that the antibacterial activity of these compounds parallels their adsorbability by plasma,[1] as well as by microörganisms.[4]

(1) B. D. Davis, *J. Clin. Invest.*, **22**, 753 (1943).

(2) D. R. Gilligan, *J. Pharmacol.*, **79**, 320 (1943).

(3) S. H. Fisher, L. Troast, A. Waterhouse and J. A. Shannon, *J. Pharmacol.*, **79**, 373 (1943).

(4) E. Havinga, H. W. Julius, H. Veldstra and K. C. Winkler, "Modern Development of Chemotherapy," Elsevier Publishing Co., Inc., New York, N. Y., 1946, pp. 45–49.

Davis[1] has demonstrated that it is the albumin fraction of plasma which is primarily responsible for the binding properties. Nevertheless, no investigations have been made of the formation of complexes between sulfonamides and crystallized serum albumin. Such a study with purified serum protein would be highly desirable, particularly since it would then be possible to apply a physicochemical analysis,[5] to the binding data and thereby to correlate the energy of binding with structural features in the drugs.

Experimental

Reagents.—Crystallized bovine serum albumin was obtained from Armour and Company. As in previous work,[5] corrections for water content were made by heating a

(5) I. M. Klotz, F. M. Walker and R. B. Pivan, THIS JOURNAL, **68**, 1486 (1946).

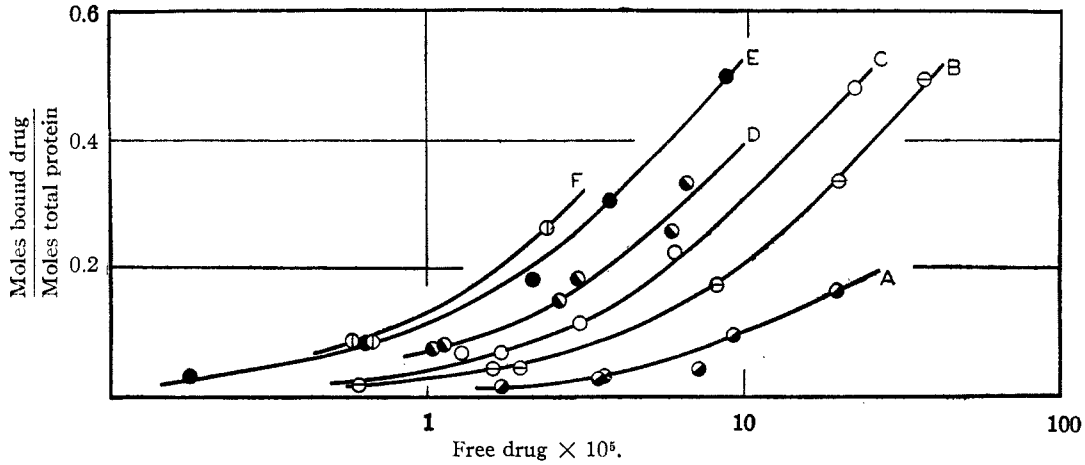


Fig. 1.—Binding of sulfonamides by bovine serum albumin in a phosphate buffer at *p*H 7.6 and 5°: A, sulfanilamide; B, sulfapyridine; C, N¹-acetylsulfanilamide; D, 5-sulfanilamido-3-methyl-isoxazole; E, 2-sulfanilamido-5-chloropyridine; F, N¹-benzoylsulfanilamide.

small sample in an oven at 110° until constant weight was attained.

Samples of the sulfonamides were kindly supplied by Drs. R. O. Roblin, Jr., and P. H. Bell of the American Cyanamid Company.

Dialysis Experiments.—The general procedure was the same as that described in earlier work.[5] Cellophane bags, prepared from commercial sausage casing, were filled with a measured amount of the protein solution of approximately 4% concentration. The bags were immersed in a solution of the sulfonamide and allowed seventy-two hours in a cold room (*ca.* 5°) for the attainment of equilibrium. For each sulfonamide concentration a control tube was also prepared which differed from the primary tube only in that the former contained buffer rather than a protein solution inside the bag.

Upon the attainment of equilibrium the sulfonamide concentrations *outside* the bags in the primary and control tubes, respectively, were analyzed. Though the compounds under investigation have no color, the absorption of ultraviolet light[6,7] may be used as an analytical method. These absorption measurements were made with a Beckman spectrophotometer using silica cells of 1 cm. thickness. The wave length of the maximum absorption of the compound was used in the analysis of the particular sulfonamide under investigation.

The calculation of the amount of bound sulfonamide per mole of total protein is essentially the same as in the example described in previous work.[6] In this investigation, however, the concentration of protein was much higher than in previous studies and hence the Donnan effect must be considered. An examination of the literature[8] reveals that the Donnan effect for serum, containing about 7% protein, would produce a ratio of monovalent anion concentrations outside and inside the bag, respectively, of about 1.02. Since the ratio is approximately proportional to the concentration of protein[8] the Donnan effect in the experiments described in this paper should produce a sulfonamide anion concentration outside the bag about 1% greater than inside.

This 1% Donnan correction on the anion concentrations would be completely negligible in the experiments with sulfanilamide and sulfapyridine, for only a small fraction of either of these compounds would be in the ionized form at the *p*H's used (7.6). For the other sulfonamides, the correction has been made though it is actually within experimental error.

In these experiments it was difficult to reproduce binding values to better than about 5%, perhaps because of very minute leakages from the bag containing the protein. While such leakages would not significantly affect the quantity of protein within the bag, it might introduce appreciable errors in the ultraviolet-absorption readings because bovine serum albumin has a strong peak in the neighborhood of those for the sulfonamides. Thus with a specific extinction coefficient, $E^{1\%}_{1\,cm.}$, of about 5 near 2800 Å.[9] the loss of as little as 0.004% protein from the liquid inside the bag (10 cc.) to that outside (20 cc.) would produce an increase in optical density of about 0.01 and hence an error of 1 to 5% in the readings (generally in the range of 0.2 to 0.8).

Results and Discussion

The binding data for six sulfonamides are summarized in Fig. 1. The method of presentation, average number of moles of bound drug per mole of total protein, *r*, *versus* the logarithm of the concentration of the free drug, log (A), is that most convenient for comparative studies of binding ability and for theoretical analysis in terms of the principles of mass action.

The equilibrium constants for the formation of the respective sulfonamide complexes may be calculated by methods previously described.[5] As is evident from Fig. 1, the average number of bound drug molecules per protein molecule does not exceed 1 over the region investigated. For purposes of comparison, then, it is sufficient to calculate k_1, the equilibrium constant for the formation of the first complex in each case. The statistical relation described previously (Equation (4) in reference (5))

$$k_i = \frac{n - (i-1)}{i} k \qquad (1)$$

may be reduced to

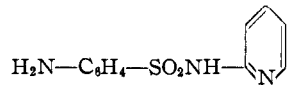
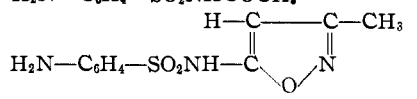
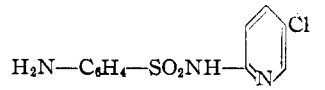
$$k_1 = nk \qquad (2)$$

for the first equilibrium constant. Since the concentration of higher complexes must be small,

(6) J. M. Vandenbelt and L. Doub, THIS JOURNAL, **66**, 1633 (1944).
(7) I. M. Klotz and D. M. Gruen, *ibid.*, **67**, 843 (1945).
(8) F. C. McLean and A. B. Hastings, *J. Biol. Chem.*, **108**, 285 (1935).
(9) A. B. Lerner and C. P. Barnum, *Arch. Biochem.*, **11**, 505 (1946).

TABLE I

COMPLEX FORMATION AND RELATED PROPERTIES FOR SOME SULFONAMIDES

Compound	Formula	pK_a[10]	Per cent. in anionic state	Association[a] constant, k_1	ΔF_1^0 cal./ mole
Sulfanilamide	$H_2N—C_6H_4—SO_2NH_2$	10.43	0.2	0.89×10^3	3740
Sulfapyridine		8.43	14	2.5×10^3	4320
N^1-Acetylsulfanilamide	$H_2N—C_6H_4—SO_2NHCOCH_3$	5.38	99	4.2×10^3	4600
5-Sulfanilamido-3-methyl isoxazole		4.2	100	7.8×10^3	4950
2-Sulfanilamido-5-chloropyridine		(7.15)[b]	80	13×10^3	5230
N^1-Benzoylsulfanilamide	$H_2N—C_6H_4—SO_2NHCOC_6H_5$	4.57	100	16×10^3	5350

[a] The buffer solution was at a pH of 7.6, and at a temperature of 5°. [b] The value for 2-sulfanilamido-5-bromopyridine has been used since data for the chloro compound are lacking.

electrostatic factors may be neglected, and the expression

$$\frac{1}{r} = \frac{1}{nk}\frac{1}{(A)} + \frac{1}{n} \qquad (3)$$

may be used as a reasonable approximation to the binding data. Thus from the slope of a graph of moles total protein per mole bound drug, $1/r$, *versus* the reciprocal of the concentration of free drug, $1/A$, one may evaluate the product nk for use in equation (2) in determining k_1. These slopes have been evaluated for the six drugs investigated and the equilibrium constants and free energy changes calculated for the association reaction

$$P + S = PS \qquad (4)$$

where P represents the protein molecule and S the sulfonamide. The results, together with acidity constants, pK_a's, taken from Bell and Roblin[10] have been assembled in Table I.

It is evident from these calculations that the binding energy increases with decreasing pK_a in the first three compounds in the table. This behavior is undoubtedly an expression of the increased concentration of anions as the pK_a decreases. That anions combine more strongly with proteins than do the corresponding non-ionized acids has been evident from other work.[11] That the sulfonamide ion is also the active species in bacteriostasis has been suggested previously.[12,13]

The difference in binding energy between sulfapyridine and 2-sulfanilamido-5-chloropyridine also seems attributable to the difference in anion concentration. If one takes into account the 5.7-fold higher anion concentration of the latter compound, it is evident that its binding energy should be approximately 960 calories (RT ln 5.7) higher than that of sulfapyridine. The observed difference in binding energy is 910 calories.

On the other hand in comparing the binding energies of N^1-acetylsulfanilamide and N^1-benzoylsulfanilamide, for which the anion concentrations in solutions of pH 7.6 are substantially identical, it is evident that another factor must enter. It seems likely that the additional stabilization energy of 750 calories for the albumin complex with benzoylsulfanilamide may be attributed to the stronger van der Waals forces which would be obtained with a phenyl group as compared to a methyl group. A similar effect has been noted by Gilbert[14] in studies of adsorption by wool where the phenyl group contributes about 1340 calories to the free energy change for the process, and the methyl group approximately 640 calories, a difference of 700 calories.

The relatively low binding energy for the isoxazole, as compared with the chloropyridine, for example, may be due to two factors. The van der Waals energy of the former is probably smaller because the number of atoms (excluding hydrogens) in it is fewer. Furthermore, the lower pK_a of the isoxazole would tend to reduce its ability to combine with the protein, if one adopts the Lewis generalized acidity concept discussed previously.[15] The loss in binding energy with decreasing pK_a in the sulfonamides seems to be small, however. If one corrects the binding energy of the chloropyridine to 100% anion concentration by adding RT ln (100/80) (130 calories), the difference between its ΔF_1^0 and that for the isoxazole is still only 400 calories for a change of 3 units in pK_a.

These investigations were supported by grants from the Lederle Laboratories Division of the American Cyanamid Company and from the Office of Naval Research.

(10) P. H. Bell and R. O. Roblin, Jr., THIS JOURNAL, **64**, 2905 (1942).

(11) I. M. Klotz, *ibid.*, **68**, 2299 (1946).

(12) C. L. Fox, Jr., and H. M. Rose, *Proc. Soc. Exp. Biol. Med.*, **50**, 142 (1942).

(13) F. C. Schmelkes, O. Wyss, N. C. Ludwig and F. B. Strandskov, *ibid.*, **50**, 145 (1942).

(14) Quoted by E. K. Rideal, *Trans. Faraday Soc.*, **39**, 368 (1943).

(15) I. M. Klotz, *Science*, **98**, 62 (1943).

Summary

Quantitative measurements have been made of the binding of sulfanilamide, sulfapyridine, N^1-acetylsulfanilamide, 5-sulfanilamido-3-methylisoxazole, 5-sulfanilamido-5-chloropyridine and N^1-benzoylsulfanilamide, respectively, by crystallized bovine serum albumin. Binding energies have been calculated and have been correlated with the structures of the compounds.

EVANSTON, ILLINOIS RECEIVED NOVEMBER 1, 1947

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF THE OHIO STATE UNIVERSITY]

The Synthesis of Some Cyclopropane and Spirane Hydrocarbons[1]

BY R. W. SHORTRIDGE, R. A. CRAIG, K. W. GREENLEE, J. M. DERFER AND C. E. BOORD

This paper describes an extension of the Gustavson method[2,3,4] for the synthesis of cyclopropane and its derivatives. Three 1,1-dialkylcyclopropanes have been prepared; two of these compounds are new, and improved physical properties were determined for the third. The method has been applied to the preparation of spiranes containing a cyclopropane ring and provides an easy, straightforward way of producing this type of hydrocarbon in quantity and in a good state of purity. Cleavage of the cyclopropane ring by hydrogen has been investigated.

1,1-Dimethylcyclopropane has been produced from *sym*-dibromoneopentane most recently by Whitmore[5] and his co-workers. Isobutyraldehyde was condensed with formaldehyde in the presence of potassium hydroxide to obtain 2,2-dimethyl-1,3-propanediol which was converted to the corresponding dibromide by the action of phosphorus tribromide; the cyclization was accomplished with zinc in molten acetamide (Hass–McBee[6] procedure). In the present work the intermediates were synthesized by the same series of reactions, but the dibromide was cyclized by zinc in aqueous ethanol. The hydrocarbon was obtained in 96% yield (based on distilled dibromide), and it froze over a 0.4° range; physical properties were determined on a center fraction from the distillation of this product at about 10-plate efficiency.[7]

1,1-Diethylcyclopropane and 1-ethyl-1-butylcyclopropane were prepared by an analogous series of reactions. The yields from the dibromides were 92 and 94%, respectively; as in the case of 1,1-dimethylcyclopropane, the initial product was of high purity.[8] These two compounds have not been described previously.

TABLE I

		B. p., °C. (760 mm.)	F. p., °C.	d^{20}_4	$n^{20}D$
1,1-Dimethylcyclopropane	This work	20.63	−108.96	0.6589	1.3668
	Whitmore[5]	19.9	−108.4 to −107.3	....	....
1,1-Diethylcyclopropane		88.67	−105.91	.7318	1.4042
1-Ethyl-1-butylcyclopropane		140.41	−102.68	.7559	1.4183

Few spiranes have been synthesized. In all reported preparations where closure of the second ring was not the final step, the over-all yields have been no more than a few per cent. Recently spiropentane has been identified as a product from the reaction of pentaerythrityl tetrabromide with zinc[9,10] the yield being in the neighborhood of 25% based on the tetrabromide; this is the highest yield reported for any spirane. We have applied the Gustavson reaction to the preparation of spiro(2.5)octane and 4-methylspiro(2.5)octane. This work provides a general method for the preparation of spiranes containing a cyclopropane ring and is limited only by the availability of cyclic aldehydes which contain the desired configuration and which will undergo the methylol reaction to yield *gem*-dicarbinols. The yields are good.

Spiro(2.5)octane was prepared in the following way: 3-Cyclohexene-1-carboxaldehyde was prepared by condensing acrolein with butadiene (Diels–Alder). The adduct, an unsaturated alde-

(1) The material in this paper is taken from three sources: (a) From the dissertation submitted by R. W. Shortridge to the Graduate School of the Ohio State University in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in 1943; present address: Midwest Research Institute, Kansas City, Missouri. This portion of the material was presented before the Organic Division at the April, 1944, meeting of the American Chemical Society. (b) From a thesis submitted by R. A. Craig to the Graduate School of The Ohio State University in partial fulfillment of the requirements for the Degree of Master of Science; present address: The Ohio State University. This portion of the material was presented before the Organic Division at the April, 1946, meeting of the American Chemical Society. (c) From the experimental work of the American Petroleum Institute Research Project 45 which is administered by The Ohio State University Research Foundation.

(2) Gustavson, *J. prakt. Chem.*, **36**, 300 (1887).

(3) Gustavson and Popper, *J. prakt. Chem.*, **58**, 458 (1898).

(4) This reaction has been called the Freund reaction on occasion. However, reference to the original literature shows that although Freund (*Monatsh.*, **2**, 642 (1881)) was the first to make cyclopropane itself, he used an extension of the Wurtz reaction, and therefore had no claim to the method of ring closure which employs zinc in the presence of protonic solvent. This method was first reported by Gustavson[2] in 1887 by a paper entitled: "Concerning a New Method of Preparation of Trimethylene." Gustavson and Popper[4] extended this method to the preparation of substituted cyclopropanes.

(5) Whitmore, Popkin, Bernstein and Wilkins, THIS JOURNAL, **63**, 124 (1941).

(6) Hass, McBee, Hinds and Glusenkamp, *Ind. Eng. Chem.*, **28**, 1178 (1936).

(7) All plate ratings given in this paper represent estimates of column efficiency under actual operating conditions.

(8) Using this method, the American Petroleum Institute Research Project No. 45 has prepared four liters of 1,1-dimethylcyclopropane and five liters of 1,1-diethylcyclopropane, both in a state of high purity.

(9) Murray and Stevenson, THIS JOURNAL, **66**, 812 (1944).

(10) Slabey, *ibid.*, **68**, 1335 1(946).