Infrared spectrograms of the isomers were determined on a Perkin-Elmer Infrared Spectrometer, Model 12A. These spectra (Figs. 6, 7, 8, and Table II) are seen to confirm the proof of structure by chemical means, the strong absorption bands characteristic of ortho occurring at 13.3 microns, of meta at 12.6 and 14.2 microns, and para at 12.0 microns. The isopropyl group is evident from the doublet in the region of 7.3 microns.

The mass spectra of the isomers, obtained on a Consolidated Engineering instrument, are shown in Table III. As would be expected no significant differences are seen in the mass spectra of the individual isomers.

TABLE III

MASS SPECTRA OF THE ISOMERIC DIISOPROPYLBENZENES

Mass	*o*-Diisopropylbenzene	*m*-Diisopropylbenzene	*p*-Diisopropylbenzene	*n*-Butane
27	31.4	44.9	38.0	
28	5.34	3.29	5.57	275
29	14.5	9.15	7.60	346
39	23.5	22.3	21.5	124
40	2.48	3.81	2.32	
41	51.8	48.8	41.8	239
42	2.82	2.45	2.58	102
43	23.1	85.6	59.5	826
44	0.80	3.01	1.97	
51	13.7	12.8	12.2	
52	3.88	3.79	3.49	
53	8.25	7.48	7.58	
58	4.08	0.75	1.61	100.0
63	7.03	7.36	6.01	
64	7.67	5.65	5.12	
65	15.3	12.3	10.9	
66	6.87	11.3	5.87	
77	23.0	24.3	22.7	
78	8.13	8.92	8.32	
79	20.2	15.1	13.0	
91	82.1	64.6	59.7	
92	6.67	5.34	4.92	
95–98[a]	4.75	0.64	0.56	
103	10.8	13.8	12.8	
104	4.48	5.79	6.06	
105	32.2	64.7	51.0	
106	2.72	5.01	4.18	
115	25.1	14.9	14.8	
116	11.1	7.79	9.03	
117	18.4	8.85	25.6	
118	4.28	3.72	5.31	
119	115	111	96.6	
120	8.96	10.4	8.20	
131	14.6	10.4	18.3	
132	2.1	2.4	13.5	
147	281	283	342	
162	100.0	100.0	100.0	
Sensitivity	23.0	22.0	23.3	4.73

[a] Metastable peak.

Acknowledgment.—Acknowledgment is hereby made to Barton Zieber for the infrared spectra and to C. W. Warfield for much of the laboratory manipulation.

Summary

The isolation of the three isomeric diisopropylbenzenes from a high boiling propylene-benzene alkylate is described. The most important physical properties and the relative concentrations of the isomers in the alkylate are listed. Infrared, ultraviolet, and mass spectra also are given, the former confirming the chemical evidence of the structure of the compounds isolated.

PHILADELPHIA, PA. RECEIVED JUNE 7, 1947

[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF NORTHWESTERN UNIVERSITY]

The Thermodynamics of Metallo-protein Combinations. Copper with Bovine Serum Albumin

BY IRVING M. KLOTZ AND HENRY G. CURME

The importance of metal ion–protein complexes in biological systems has long been recognized, and numerous studies have been made of the extent of combination of such cations with proteins.[1,2,3] Unfortunately most of these investigations have utilized protein preparations of questionable homogeneity and have correlated their quantitative data in terms of equations which are essentially empirical in nature. With the recent availability of crystallized plasma proteins[4] it has seemed opportune to make a careful quantitative study of metal complexes with pure proteins and to treat the data obtained in terms of relations similar to those used in describing the multiple equilibria between proteins and organic anions.[5]

(1) J. H. Northrup and M. Kunitz, *J. Gen. Physiol.*, **7**, 25 (1924); **9**, 351 (1925); **11**, 481 (1928).

(2) D. M. Greenberg, in M. L. Anson and J. T. Edsall, "Advances in Protein Chemistry," Volume I, Academic Press, New York, N. Y., 1944, pp. 121–151.

(3) C. L. A. Schmidt, "The Chemistry of the Amino Acids and Proteins," Charles C. Thomas, Springfield, Ill., 1945, pp. 293–296, 746–762, 1091–1092, 1213–1214.

(4) E. J. Cohn, J. L. Oncley, L. E. Strong, W. L. Hughes, Jr., and S. H. Armstrong, Jr., *J. Clin. Invest.*, **23**, 417 (1944).

Experimental

Apparatus.—The extent of binding of cupric ions by bovine serum albumin was measured by the equilibrium dialysis technique described previously.[5] However, the apparatus was modified slightly so that a bead could be suspended by a glass thread within the bag containing the protein solution. With the bead to facilitate stirring within the bag and with a mechanical shaker to agitate the outside solution, the time for the attainment of equilibrium was reduced materially.

Time Required to Attain Equilibrium.—With the modified apparatus the dialysis tubes reached equilibrium in

(5) I. M. Klotz, F. M. Walker and R. B. Pivan, THIS JOURNAL, **68**, 1486 (1946); **69**, 1609 (1947).

less than six hours. This was true whether protein solution or buffer was inside the bag, as is illustrated by the data in Table I. For the work carried out at 25° this shaking method was used, therefore. For the studies at 0° the dialysis tubes were maintained in an ice-water-bath, with occasional shaking by hand, for a period of seventy-two hours. In both cases, temperatures were maintained within ±0.05°.

TABLE I

TIME REQUIRED TO ATTAIN EQUILIBRIUM

Time in hours	Free Cu^{++} in blank bag	Free Cu^{++} in protein bag
0	0.0048	0.0048
6	.00282	.00195
10	.00282	.00195
22	.00283	.00195

Analytical Methods.—Analyses for cupric-ion concentrations were made by one of two methods, depending on the concentration range under investigation. In solutions of 0.001 to 0.01 molar the triethanolamine method of Yoe and Barton[6] was used. Two ml. of a 25% solution of triethanolamine in water was added to 10 ml. of the cupric ion solutions in acetate buffer and the absorption of light at 6460 Å. was measured with a Beckman spectrophotometer. In the dilute region of cupric ion concentrations, *i. e.* below 0.001 molar, the absorption of ultraviolet light by the cupric ions themselves was used to determine the concentration of cations.

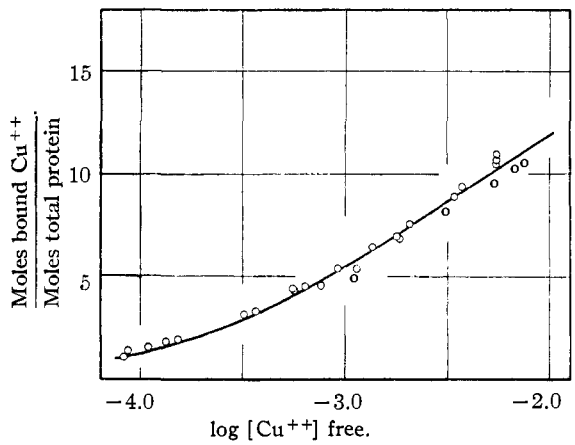

Fig. 1.—Binding of cupric ions by bovine serum albumin at 0°; *p*H of 4.83 at 25°.

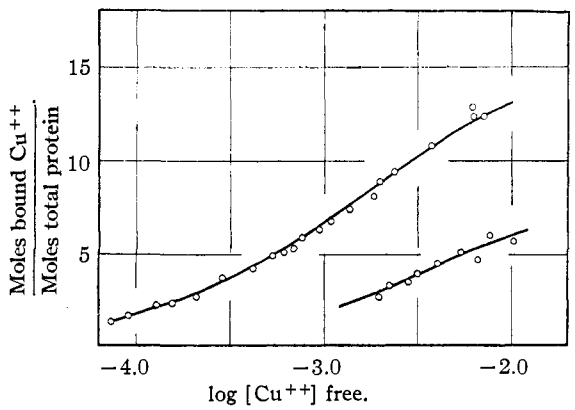

Fig. 2.—Binding of cupric ions by bovine serum albumin at 25°: upper curve at *p*H 4.83; lower curve at *p*H 4.00.

(6) J. H. Yoe and C. J. Barton, *Ind. Eng. Chem., Anal. Ed.*, **12**, 456 (1940).

Materials.—Bovine serum albumin was Armour crystallized product. Corrections for water content were made by heating a small sample in an oven at 110° until constant weight was attained. Solutions of approximately 2% protein content were used. The copper salts employed were of analytical reagent grade and were used without further purification. Triethanolamine was an Eastman product.

The buffer solution used for the experiments near *p*H 5 was composed of 0.0357 molar acetic acid and 0.0643 molar sodium acetate. The same concentration of sodium acetate was used in the buffer of *p*H 4 but the acetic acid was increased to 0.2455 molar. The same concentration of acetate was used at both *p*H's in order to keep the ionic strengths equal in both solutions.

Results and Calculations

The extent of binding of cupric ions by bovine serum albumin has been calculated from the difference in the concentrations of free copper in a control tube containing buffer inside the bag and one containing protein. The details of such a calculation have been outlined previously.[5] The degree of binding has been represented in graphs of the average number of bound ions per molecule of protein versus the logarithm of the concentration of the free cupric ion, as is illustrated in Figs. 1 and 2. It is immediately evident, as has been observed previously for organic anions,[5] that the bovine albumin molecule is capable of binding many cupric ions even at relatively low concentrations of free copper.

Reversible Nature of the Binding.—Before considering the application of the laws of equilibirum to these observations it has seemed desirable to establish the reversible nature of the process under examination. To settle this point the experiment outlined in Table II was carried out. The dialysis bag was removed from a solution in which equilibrium had been attained between protein and free Cu^{++} at a final concentration of 9.18×10^{-4} *M*. This bag, containing an average of 6.30 bound ions per molecule of protein, was then placed in a solution of pure buffer and the bound copper allowed to dialyze out until a second equilibrium was established. From the volumes of the solutions used, and the concentration of free Cu^{++} at the end of the second equilibrium, it was possible to determine the average number of bound ions under the new conditions. This observed value, 4.39, is in excellent agreement with that of 4.60 which may be read from Fig. 2. The binding of Cu^{++} by bovine albumin thus seems to be a reversible process.

TABLE II

REVERSIBILITY OF BINDING

	First equilibrium	Second equilibrium
Free Cu^{++}, *M*	9.18×10^{-4}	4.98×10^{-4}
Number of bound ions per molecule of protein	6.30	4.39 (observed) 4.60 (from graph)

Method of Representation of the Data.—Under these circumstances it is permissible to

represent the series of equilibria involved by equations of the form

$$PCu_{i-1} + Cu^{++} = PCu_i \qquad (1)$$

where PCu_{i-1} and PCu_i represent protein–copper complexes with $i-1$ and i cupric ions, respectively. The number i may vary from 1 to n, the maximum number of bound cations. The moles of bound cation per mole of total protein, represented by r, would be obtained from the expression

$$r = \frac{\sum_{i=1}^{n} i \left(\prod_{j=1}^{i} k_j \right) (Cu^{++})^i}{1 + \sum_{i=1}^{n} \left(\prod_{j=1}^{i} k_j \right) (Cu^{++})^i} \qquad (2)$$

where the k's represent the equilibrium constants corresponding to each of the reactions of (1). Thus the calculation of the moles of bound ion per mole of total protein depends on a knowledge of these n equilibrium constants.

For the ideal situation, based on a model of a spherical protein molecule and assuming the absence of electrostatic interactions between successively bound ions, the equilibrium constants bear a simple relation to each other, given by the equation

$$k_i = \frac{n - (i-1)}{i} k \qquad (3)$$

in which k is an intrinsic constant which depends on the nature of the protein and of the ion which is bound. When equation (3) is applicable, equation (2) may be simplified appreciably and the binding data may be correlated by the linear expression

$$\frac{1}{r} = \frac{1}{kn} \frac{1}{(Cu^{++})} + \frac{1}{n} \qquad (4)$$

It has been shown previously[7] that the binding of calcium ions by casein may be represented adequately by an equation of the form of (4). For complexes between copper and bovine albumin, however, this linear expression has been found not to fit the observed data.

In similar circumstances in studies of the binding of organic anions by proteins,[5] deviations from the simple statistical relation have been attributed to electrostatic interactions between successively bound ions and have been accounted for quantitatively by assuming that they may be estimated from the equations of the simple Debye–Hückel theory. Thus successive equilibrium constants have been related to each other by the equation[5]

$$RT \ln (k_{i-1}/k_i) = RT \ln \left[\frac{n - (i-2)}{n - (i-1)} \frac{i}{i-1} \right] + \frac{Nz^2e^2}{D} \left(\frac{1}{b} - \frac{\kappa}{1 + \kappa a} \right) \qquad (5)$$

with the second term representing the electrostatic factor. An attempt has been made to use this equation to correlate the data on copper complexes also. For this purpose, a value of 3 Å. was used for the radius of the aqueous cupric ion[8] and 30 Å. for the serum albumin molecule. With these parameters, values of $\Delta F_{elect.}$, the second term in equation (5), were found to be 169 and 186 calories per mole at 0° and 25°, respectively, for free copper concentrations below about 0.001 molar.

Using such values for the electrostatic-interaction term, wide deviations were obtained between calculated and observed values of r. Excellent agreement between calculated and observed binding curves (Figs. 1 and 2) was obtained, however, when $\Delta F_{elect.}$, was reduced arbitrarily to 60 calories/mole at both temperatures. The possible significance of this discrepancy between the theoretical and empirical values of $\Delta F_{elect.}$ will be discussed shortly.

Using 60 calories/mole for $\Delta F_{elect.}$ and 16 for n, one can calculate the free energy of formation of each protein-cation complex from its predecessor. These free energies, together with the entropies and enthalpies which may be derived therefrom by standard thermodynamic procedures, have been summarized in Table III. Strictly speaking these thermodynamic values refer only to solutions with copper concentrations not exceeding 0.001 molar. Above this concentration the cupric salts contribute appreciably to the ionic strength of the solution and hence change $\Delta F_{elect.}$ slightly. For most purposes, however, the slight discrepancies may be neglected.

TABLE III

THERMODYNAMICS OF BINDING OF Cu^{++} BY BOVINE SERUM ALBUMIN

*p*H 4.83 at 25°; $PCu_{i-1} + Cu^{++} = PCu_i$

	ΔF^0 at 0°. cal./mole	ΔF^0 at 25°. cal./mole	ΔS^0 cal./mole/deg.	ΔH^0 cal./mole
1	−5179	−5908	29.2	2780
2	−4708	−5399	27.6	2840
3	−4391	−5058	26.7	2900
4	−4135	−4784	26.0	2960
5	−3910	−4544	25.4	3020
6	−3704	−4325	24.8	3080
7	−3509	−4117	24.3	3140
8	−3319	−3915	23.8	3200
9	−3131	−3715	23.4	3260
10	−2941	−3513	22.9	3320
11	−2746	−3305	22.4	3380
12	−2540	−3086	21.8	3440
13	−2315	−2846	21.2	3500
14	−2059	−2572	20.5	3560
15	−1742	−2231	19.6	3620
16	−1271	−1722	18.0	3680

These free energy quantities may be correlated also in the form of the equations

$$\text{At } 0°: \Delta F^0_i = -5179 + \sum_{j=2}^{j=i} RT \ln \frac{n - (j-2)}{n - (j-1)} \frac{j}{j-1} + (i-1)\phi_{elect.} \qquad (6)$$

(7) I. M. Klotz, *Arch. Biochem.*, **9**, 109 (1946).

(8) J. Kielland, THIS JOURNAL, **59**, 1675 (1937).

$$\text{At } 25°: \Delta F^0_i = -5908 + \sum_{j=2}^{j=i} RT \ln \frac{n-(j-2)}{n-(j-1)} \frac{j}{j-1} + (i-1)\phi_{\text{elect.}} \quad (7)$$

where $\phi_{\text{elect.}}$ is taken as 60 calories/mole at each temperature. From these equations it is possible to deduce the following expressions for the entropy and enthalpy changes

$$\Delta S^0_i = 29.2 - \sum_{j=2}^{j=i} R \ln \frac{n-(j-2)}{n-(j-1)} \frac{j}{j-1} \quad (8)$$

$$\Delta H^0_i = 2780 + (i-1)\phi_{\text{elect.}} \quad (9)$$

It should be pointed out that in the general case the equations for ΔS^0_i and ΔH^0_i would also contain a term involving the factor $\partial\phi_{\text{elect.}}/\partial T$. However, in the present situation the data can be represented adequately with the same value of $\phi_{\text{elect.}}$ at both temperatures so the temperature coefficient of the electrostatic term vanishes.

Effect of *p*H on Binding.—Since the *p*H dependence of the binding ability reflects on the nature of the groups involved in the bond, a few experiments were carried out at a *p*H of 4.0. These data are compared in Fig. 2 with the corresponding results at *p*H 4.8 at the same temperature. It is immediately evident that the maximum number of binding groups has been decreased substantially in solutions of higher acidity.

Discussion

Thermodynamics of the Complex-formation.—Both the table and equations showing the dependence of ΔF^0 on i illustrate the general trend downward in stabilization energy as the number of bound ions increases. The equations (6) and (7), in addition, show explicitly the basic sources of this trend—the contributions of the statistical and electrostatic terms, respectively, to the free-energy change. In the same connection it is of interest to note that the expression for the entropy, equation (8), contains the statistical term and lacks the electrostatic one, whereas the expression for the enthalpy, equation (9), includes an electrostatic contribution but no statistical one. Thus if no electrostatic interactions were involved, the enthalpy change, ΔH^0_i, would be the same for each successive complex.

It is also of interest to note that it is $T\Delta S$ term which supplies the necessary energy to make ΔF negative in the binding process. Thus from Table III it is evident that with positive values for the heat of binding, complex-formation between albumin and cupric ions would not be favored, were it not for the relatively large values of ΔS. The magnitude of the entropy changes probably indicates that the transfer of a Cu^{++} ion from the solvent to the protein molecule is accompanied by the release of several "frozen" water molecules and acetate ions.

Magnitude of the Electrostatic Effect.—It has been mentioned above that a value of 60 calories has been used for $\phi_{\text{elect.}}$ rather than 186 calories as would be expected from available molecular parameters and the electrostatic portion of equation (5). The basis of this large discrepancy is not clear. One source might be the use of a spherical model for the protein molecule instead of an ellipsoidal one. To estimate the magnitude of this difference, a calculation has been made of the electrostatic charging energy (at infinite dilution) of an ellipsoid of revolution with a major axis[4] of 150 Å. and a minor axis of 38 Å., and the value obtained has been compared with that of a sphere with a diameter of 60 Å. Though both these models encompass the same volume, the charging energy of the ellipsoid is 84% that of the sphere. The use of an ellipsoidal model, therefore, would change $\phi_{\text{elect.}}$ in the correct direction. Nevertheless, the extent of the decrease is not nearly sufficient to account for the observed drop from 186 to 60, or down to almost 30% of the spherical value.

It should be mentioned, of course, that the use of the Debye–Hückel expression to obtain the electrostatic portion in equation (5) is not really justified from a molecular point of view, for the protein molecule has discrete charges distributed along its surface as well as in its interior. In the absence of detailed information on the distribution of these charges it would be difficult to derive any convenient expression for further approximation of the electrostatic effect. Nevertheless, in connection with calculations at higher copper concentrations, where the cupric ions make an appreciable contribution to the ionic strength, it has been found convenient to use the Debye–Hückel expression and to take account of its inadequacy by increasing the magnitude of the dielectric constant to 350. This is a procedure similar to that used by Kirkwood and Westheimer,[9] though we do not have a corresponding theoretical justification. Using this higher "effective" dielectric constant at higher copper concentrations (above 10^{-3} *M*) one finds good agreement between the observed and calculated values of the extent of binding. Nevertheless, this agreement cannot be used to validate the approach because the binding equations are not sufficiently sensitive to small changes in the electrostatic contribution.

Nature of the Cation–Protein Bond.—The decrease in extent of binding at *p*H 4.0 as compared to that observed at *p*H 4.8 (Fig. 2) indicates that anionic carboxylate groups on the protein molecule are involved in bond formation with the cation. Such behavior would be in line with the many observations of complex-formation between cupric ions and carboxylic acids, including amino acids,[10] where similar *p*H dependence has been found. That the cation–protein linkage is through the carboxyl group is indicated also by the absorption spectrum of cu-

(9) J. G. Kirkwood and F. H. Westheimer, *J. Chem. Phys.*, **6**, 506 (1938).

(10) H. Borsook and K. V. Thimann, *J. Biol. Chem.*, **98**, 671 (1932).

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.

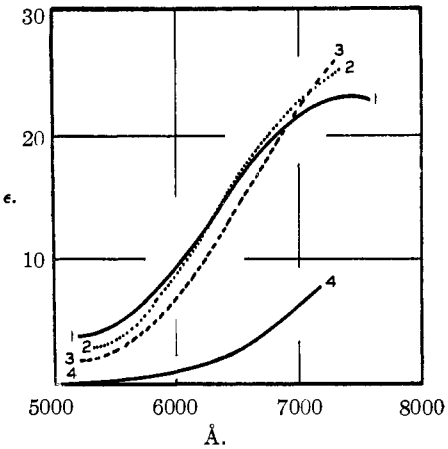

Fig. 3.—Absorption spectra of some copper complexes: 1, cupric ions with 2% bovine serum albumin at *p*H 4.23; 2, copper acetate in alcohol[10]; 3, cupric ions with glycine[10]; 4, cupric chloride *p*H 4.66.

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).