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Sedimentation Equilibrium Studies with Chymotrypsinogen A in Solution at pH 7.9 and $I = 0.03$*

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ABSTRACT: By using the sedimentation equilibrium experiment, it has been found that chymotrypsinogen A undergoes an association reaction when it is dissolved in Veronal buffer at pH 7.9 and at an ionic strength of 0.03. A quantitative evaluation of the data would seem to indicate that under these conditions, the reaction is one of indefinite self-association. However, while every known precaution has been taken to obtain data of high precision, the discrete mechanism of a monomer–dimer–trimer process cannot be completely eliminated. A reason is provided to account for the fact that this discrete association model does almost as well in reproducing the experimental data as does the random association mechanism.

In a recent survey report Nichol (1968) has given consideration to the velocity and equilibrium sedimentation behavior of chymotrypsinogen A in the isoelectric region in glycine-buffered solutions. For several reasons it was not possible at the time to complete a really quantitative treatment with reference to a particular nonideal associating system. For instance, an analysis of the data in terms of a monomer–dimer model lacked uniqueness; certain other mechanisms served about as well to account for the observations.

There is no doubt, however, that given proper solution conditions, association processes do exist. Thus more definitive information about the nature of the association was sought. Scrutiny was restricted to the sedimentation equilibrium ex-

* From the Department of Chemistry, University of Wisconsin, Madison, Wisconsin 53706. *Received February 27, 1969.* This research received its financial support from the National Science Foundation (Grant GB 6063).

periment and additional extended measurements of the highest available precision were made on chymotrypsinogen A in Veronal buffer solutions at a pH slightly below the isoelectric point.

In a number of ways chymotrypsinogen A is an ideal substance for study. It has been established that it exists as monomer in certain media, it can be prepared in substantially homogeneous condition, its complete amino acid sequence has been worked out, and its three-dimensional X-ray analysis is well along toward completion. Thus, the molecular weight of the monomer is a well-established quantity, an important item for the necessary calculations.

The experimental methods and techniques, along with the evaluation of the quantities $M_{w(c)}^{app}$, the apparent weight-average molecular weight at the radial distance, r, corresponding to the concentration $c = c(r)$, and the concentration $c(r)$ are those already described by Albright and Williams (1968). It will be seen, however, that the interpretations of these new data are more involved, simply because the reaction mechanisms are more complicated. The data and their interpretation will be described in some detail in the text to follow. The results of the calculations indicate that under the conditions of the experiments chymotrypsinogen A seems to undergo an indefinite self-association ("random polymerization"), but it is also shown that until even more exacting data can be made available such discrete mechanisms as a monomer–dimer–trimer process cannot be really ruled out.

Experimental Section

Chymotrypsinogen A. This protein was obtained as a chromatographically homogeneous material (lot no. CGC 762, Worthington Biochemical Corp.). Repeated molecular weight determinations by sedimentation equilibrium methods showed stock solutions of the protein to be stable for at least a week; hence the preparation was used without further treatment. The same material was used by Nichol; it had been tested by him in several ways.

Veronal Buffer. A buffer of ionic strength $I = 0.03$ was prepared by weighing appropriate amounts of reagent grade sodium chloride and 0.602 N sodium hydroxide into approximately the desired volume of deionized water and adjusting the pH to 7.90 with 5,5-diethybarbituric acid, again of reagent grade. The final volume adjustment was insufficient to change the pH, as measured by a Beckman Model G pH meter.

Preparation of Solutions. Stock solutions were obtained by adding chymotrypsinogen A to 3 ml of Veronal buffer and dialyzing against five successive 300-ml portions of buffer for a total of 24 hr, then against 500 ml of buffer for 12 hr. All protein solutions were prepared by diluting fresh stock solutions with the last portion of buffer which had been brought to dialysis equilibrium with the protein stock solution.

Protein Concentrations. Concentrations of solutions for each experiment were calculated from absorption measurements at 282 mμ of appropriately diluted solutions, using a Beckman DB spectrophotometer and an extinction coefficient of 20.3 dl/g cm (Brandts and Lumry, 1963). Dilutions were made by weight, taking into account the difference in solvent and solute density as calculated from the equation, $\rho = \rho_0 + (1 - \bar{v}\rho_0)c/100$, where c is in grams per deciliter. A value of 0.737 ml/g was used for the partial specific volume (Nichol, 1968).

Density Measurements. The buffer density at 25.0° was determined by using a 20-ml Ostwald pycnometer. Weighings were carried out on a Mettler B5 balance, with all readings being corrected for air buoyancy in the usual way.

Equilibrium Ultracentrifugation. All experiments were performed at 25.00 ± 0.05°, using a Spinco Model E analytical ultracentrifuge equipped with a temperature regulation unit. The 22-lb Spinco An-J rotor was used throughout, since at low speeds this heavier rotor reduces fluctuations in speed and rotor precession. Rotor speeds were calculated from odometer readings.

All equilibrium experiments were carried out with a double-sector cell equipped with a 12-mm Epon centerpiece, sapphire windows, and interference double-slit window holders. Rayleigh interference and schlieren optical systems were used. The schlieren diaphragm consisted of a phase plate with a metal line deposited at the phase-plate edge. A symmetrical double-slit upper collimating lens mask was used. An initial schlieren photograph served to determine the meniscus position and that of the cell bottom. Because of a recent finding by Adams and Lewis (1968) that a reaction may occur between some protein solutions and the bottom layering fluid, the layering fluid was omitted. (In general, this is considered to be good practice, though it does make more difficult the location of the cell position, r_b.)

The cell compartments were loaded by means of a syringe microburet with 0.12 ml of protein solution and 0.14 ml of solvent to give a solution column height of approximately 3.1 mm. Polyethylene needles were used for the insertion of the solution in order to minimize effects of protein adsorption.

The rotor speed was chosen so as to give 15–20 fringes at equilibrium except for the lowest concentrations, where only 7 and 10 fringes were obtained. The speeds used varied between 4,900 and 16,200 rpm. The time required to attain equilibrium was calculated to be about 20 hr, though in practice the duration of an experiment was at least 24 hr. The substantial attainment of equilibrium was checked by a series of Rayleigh photographs taken at about 2-hr intervals.

Rayleigh and schlieren patterns were recorded on Kodak Type II-G spectroscopic plates. The patterns were analyzed with the aid of a Gaertner plate reader. Cell base-line corrections were applied to all fringe displacement readings.

Results

Sedimentation equilibrium experiments at several different initial protein concentrations were performed and $M_{w(c)}^{app}$ *vs.* c data were then calculated over a wide range of concentrations, using the basic sedimentation equilibrium equation

$$M_{w(c)}^{app} = \frac{d(\ln c(r))}{d(r^2)} \frac{2RT}{(1 - \bar{v}\rho)\omega^2} \quad (1)$$

The concentration c is measured in grams per deciliter.

Since the ln c *vs.* r^2 plots show very little curvature, difference curves were used to obtain more accurate derivatives; *i.e.*, $\Delta \ln c(r)$, which is the difference between the ln $c(r_i)$ of the data and the ln $c(r_i)$ of a line drawn between the first and last data points was plotted *vs.* r^2. This gives an expanded plot from which one can obtain the tangents representing the difference in slope between the original curve of ln c *vs.* r^2 and

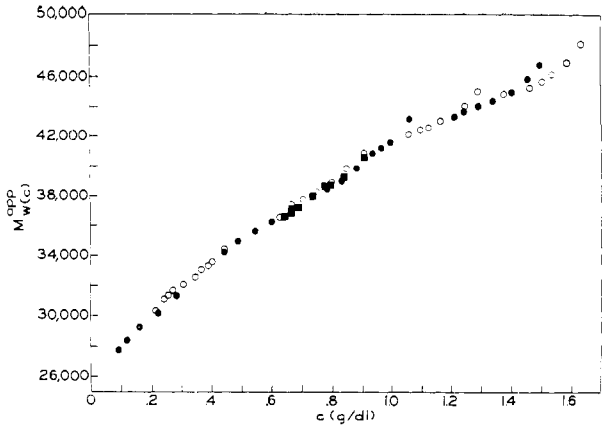

FIGURE 1: $M_{w(c)}^{app}$ *vs.* concentration. Chymotrypsinogen A in solution at pH 7.9, $I = 0.03$.

the arbitrary straight line. The $M_{w(c)}{}^{app}$ *vs.* c data so calculated are presented as points in Figure 1. A smooth curve drawn through these data points has been used in all subsequent calculations.

In order to proceed, the $M_{w(c)}{}^{app}$ *vs.* c curve must first be transformed into the reduced reciprocal function, $M_1/M_{w(c)}{}^{app}$ *vs.* c. In the present case, the monomer molecular weight is independently known from amino acid analyses to be 25,600, a value confirmed by the sedimentation equilibrium studies of LaBar (1965).

From the experimental data the apparent number-average molecular weight, $M_{n(c)}{}^{app}$, as a function of concentration, c, can be calculated according to the relation (Adams, 1965)

$$\frac{cM_1}{M_{n(c)}{}^{app}} = \int_0^c \frac{M_1}{M_{w(c)}{}^{app}} dc \qquad (2)$$

The $M_1/M_{n(c)}{}^{app}$ values were obtained for subsequent use by numerical integration, using the trapezoidal rule.

Since the exact form of the $M_1/M_{w(c)}{}^{app}$ *vs.* c and $M_1/M_{n(c)}{}^{app}$ *vs.* c curves depends upon the type of association involved, the equilibrium constant(s) for the association, and the virial coefficient, a set of equilibrium constants and virial coefficients was sought which would best predict these data and hence determine the type of association involved in the chymotrypsinogen solution at the temperature and pH conditions which were employed.

The $M_{w(c)}{}^{app}$ *vs.* c data gave no indication of leveling off at high concentrations. Thus, it appears likely that species higher than dimers are present and that perhaps the association might best be described by a random or indefinite association. As pointed out by Nichol (1968), the charges on the chymotrypsinogen A molecule are grouped in positive and negative regions and one might therefore envision a stacking of the chymotrypsinogen molecules. However, one must not overlook the possibility that the procees is limited to a relatively few species in chemical equilibrium with one another, in other words, that what may be termed a discrete self-association is operative. To carry out the comparison the data have been evaluated by using two models for the analyses: one, an indefinite or random association, and two, a monomer–dimer–trimer process. The methods for consideration of the data are clearly delineated in a recent report of Adams (1967). For the two cases we present merely a procedural outline, writing down in order the Adams equations as used in the calculations.

TABLE I: Evaluation of the Intrinsic Equilibrium Constant k for the Random Association Process ($\hat{B}M_1 = +4.0$).

C (g/ml)	C_1 from Eq 5	C_1 from Eq 6	k	$\frac{M_1}{M_{w(C)}{}^{app}}$
0.0005	0.0004785		45.35	0.9556
0.0010	0.0009146		47.73	0.9172
0.0015	0.001312		49.38	0.8838
0.0020	0.001677	0.001678	50.21	0.8544
0.0025	0.002016		50.58	0.8282
0.0030	0.002333		50.67	0.8047
0.0035	0.002631		50.55	0.7832
0.0040	0.002912	0.002912	50.38	0.7636
0.0045	0.003179		50.20	0.7457
0.0050	0.003431		50.04	0.7293
0.0055	0.003671		49.87	0.7141
0.0060	0.003899	0.003890	49.72	0.7000
0.0065	0.004117		49.58	0.6870
0.0070	0.004326		49.45	0.6748
0.0075	0.004529		49.22	0.6632
0.0080	0.004724	0.00470	49.03	0.6525
0.0085	0.004905		48.99	0.6427
0.0090	0.005079		48.99	0.6337
0.0095	0.005244		49.02	0.6253
0.0100	0.005402	0.00537	49.06	0.6175
0.0105	0.005553		49.11	0.6102
0.0110	0.005699		49.17	0.6034
0.0115	0.005839		49.23	0.5970
0.0120	0.005974	0.00595	49.28	0.5910
0.0125	0.006105		49.33	0.5853
0.0130	0.006232		49.37	0.5799
0.0135	0.006355		49.40	0.5748
0.0140	0.006474	0.00645	49.42	0.5700
0.0145	0.006591		49.43	0.5654
0.0150	0.006706		49.42	0.5610
		Av	49.58	

Random Association. Making use of the statement

$$\frac{M_1}{M_{w(C)}{}^{app}} = \frac{1}{\frac{2}{\left(\frac{M_1}{M_{n(C)}{}^{app}} - \frac{\hat{B}M_1C}{2}\right)} - 1} + \hat{B}M_1C \qquad (3)$$

the quantity $\hat{B}M_1$ is found by successive approximations. The concentration C is measured in grams per milliliter and $\hat{B}M_1C = BM_1c$.

It then becomes possible to compute the quantity $1 - kC_1$ from the equation

$$1 - kC_1 = \frac{M_1}{M_{n(C)}{}^{app}} - \frac{\hat{B}M_1C}{2} \qquad (4)$$

Here k is an intrinsic association constant. In this way the

TABLE II: Evaluation of the Equilibrium Constants K_2 and K_3 for the Monomer–Dimer–Trimer Association Process ($BM_1 = -0.01$) ($K_2 = 2k/100$, in dl/g).

c (g/dl)	c_1	K_2	K_3	$\frac{M_1}{M_{w(c)}^{app}}$
0.00				
0.05	0.04782	0.971	0.907	0.9564
0.10	0.09167	0.909	0.913	0.9175
0.15	0.1321	0.898	0.924	0.8831
0.20	0.1694	0.911	0.922	0.8530
0.25	0.2043	0.921	0.915	0.8261
0.30	0.2370	0.940	0.900	0.8018
0.35	0.2679	0.953	0.882	0.7797
0.40	0.2971	0.963	0.866	0.7595
0.45	0.3249	0.955	0.853	0.7412
0.50	0.3514	0.954	0.843	0.7245
0.55	0.3766	0.912	0.843	0.7091
0.60	0.4009	0.952	0.836	0.6951
0.65	0.4241	0.951	0.830	0.6821
0.70	0.4464	0.948	0.816	0.6702
0.75	0.4679	0.942	0.813	0.6591
0.80	0.4887	0.931	0.810	0.6488
0.85	0.5087	0.916	0.809	0.6394
0.90	0.5279	0.899	0.809	0.6307
0.95	0.5465	0.884	0.811	0.6229
1.00	0.5646	0.872	0.811	0.6153
1.05	0.5820	0.863	0.814	0.6084
1.10	0.5989	0.859	0.816	0.6019
1.15	0.6154	0.856	0.818	0.5959
1.20	0.6314	0.857	0.820	0.5900
1.25	0.6472	0.855	0.821	0.5843
1.30	0.6623	0.858	0.824	0.5793
1.35	0.6772	0.866	0.826	0.5743
1.40	0.6918	0.874	0.827	0.5695
1.45	0.7060	0.887	0.828	0.5648
1.50	0.7201	0.886	0.828	0.5603
		Av 0.908	Av 0.844	

monomer concentration C_1 is made available, since

$$C = C_1/(1 - kC_1)^2 \tag{5}$$

when $kC_1 < 1$. The concentration C_1 also may be computed for comparison by using the formula

$$C_1 = Cf_1^{app}e^{-\hat{B}M_1C} \tag{6}$$

where

$$\ln f_1^{app} = \int_0^C \left(\frac{M_1}{M_{w(C)}^{app}} - 1\right)\frac{dC}{C}$$

Values of the intrinsic constant k follow directly.

In this way the curve $M_1/M_{w(C)}^{app}$ *vs.* C may be generated from the equation

$$\frac{M_1}{M_{w(C)}^{app}} = \frac{1 - kC_1}{1 + kC_1} + \hat{B}M_1C \tag{7}$$

Values for the quantities C_1, k, and $M_1/M_{w(C)}^{app}$ are collected to form Table I. An additional significant figure has been retained to reduce rounding off error, both here and in Table II.

Monomer–Dimer–Trimer Association. Again it is necessary to obtain a quantitative description of the nonideality term, this time BM_1, with the concentrations, c, now being given in grams per deciliter. This is again achieved by a process of successive approximation, using eq 8.

$$\frac{6cM_1}{M_{n(c)}^{app}} - 5c = 2cf_1^{app}\exp(-BM_1c) + 3BM_1c^2 - \frac{1}{\left(\frac{M_1}{cM_{w(c)}^{app}} - BM_1\right)} \tag{8}$$

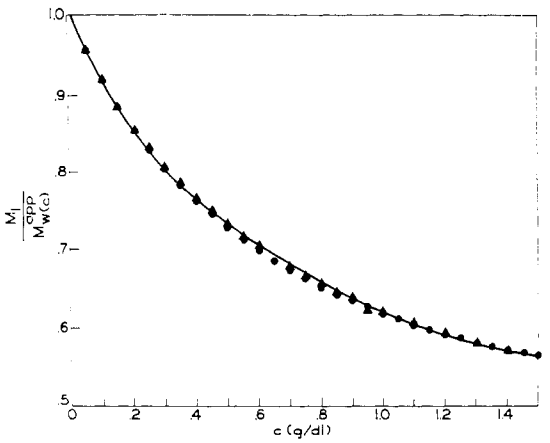

FIGURE 2: Comparisons of experimental and calculated $M_1/M_{w(c)}^{app}$ *vs. c* curves. Full line, from experimental data. (▲) For random association, with $k = 49.6$ and $\hat{B}M_1 = +4.0$. (●) For monomer–dimer–trimer association with $K_2 = 0.908$, $K_3 = 0.844$, and $BM_1 = -0.01$.

The monomer concentration, c, is then computed as before, using eq 6.

Still required are the equilibrium constants K_2 and K_3. They have their source in the expressions

$$K_2c_1^2 = 3c - 2c_1 - \frac{1}{\left(\frac{M_1}{cM_{w(c)}^{app}} - BM_1\right)} \quad (9)$$

$$c = c_1 + K_2c_1^2 + K_3c_1^3 \quad (10)$$

It is now possible to construct the curve $M_1/M_{w(c)}^{app}$ *vs. c* from the statement

$$\frac{M_1}{M_{w(c)}^{app}} = \frac{c}{c_1 + 2K_2c_1^2 + 3K_3c_1^3} + BM_1c \quad (11)$$

by using the averaged values of BM_1, K_2, and K_3 given in Table II.

Discussion

An objective of this research was to ascertain whether it is really possible to distinguish between a discrete and random protein association reaction by using the sedimentation equilibrium experiment and a mathematical analysis of the data thereby provided. However, before moving ahead to this end, it should be noted that until recently there has been a serious difficulty because plots of the quantity $M_{w(c)}^{app}$ *vs.* $c(r)$ for a number of individual experiments at several different protein (and buffer) concentrations did not exactly overlap to form a single continuous curve, as required by theory. This situation has been described in several places in the literature.

It can be noted that this problem has not been encountered in very recent investigations with either β-lactoglobulin A (Adams and Lewis, 1968) or with β-lactoglobulin B (Albright and Williams, 1968). In addition, with the present experimental results for chymotrypsinogen A solutions the merger of the several $M_{w(c)}^{app}$ *vs.* $c(r)$ curves corresponding to a number of initial protein concentrations can be said to be excellent in general, but it is evident that there does remain some uncertainty about the behavior at the higher solute concentrations.

Various causes for this general situation have been suggested, such as the use of layering oils in the ultracentrifuge cell, the composition of the cell sector pieces, effects of pressure, etc. With regard to the effect of pressure on the association, it should be negligibly small in our experiments in view of the low angular velocities and short solution column heights employed. Also, various tests conducted by Nichol (1968) indicate that the aluminum sector pieces are unlikely sources of trouble with chymotrypsinogen A solutions. We feel the main reason for the more recent generally good conformity to the theoretical requirement of a smooth over-all concentration dependence of $M_{w(c)}^{app}$ to be enhanced precision of the experimental results.

So, with the improvement of the data themselves we can look with renewed interest to the possibility of the assignment of a single type of association mechanism to the reaction which must be taking place in the solution. To this end reference to Figure 2 may be made. For its construction the reciprocal function $M_1/M_{w(c)}^{app}$, either computed directly from the data of Figure 1, or from theory, is plotted against protein concentration. It appears that the experimental data (full line) are somewhat better represented by the filled triangles, calculated by using the quantities, $k = 49.6$ and $\hat{B}M_1 = 4.0$ with the theoretical equations descriptive of the random association–mechanism, than they are by the filled circles which have been obtained by using the data $K_2 = 0.908$, $K_3 = 0.844$, and $BM_1 = -0.01$ in the expressions which describe a monomer–dimer–trimer equilibrium. It may seem to be unrealistic to be forced to utilize a negative value for the term BM_1, but even so it has to be recognized that any differentiation between the two reaction mechanisms is anything but positive and clear-cut.

For the discrete association mechanism, it appears at once (Table II) that the difference in magnitude between K_2^2 and K_3 is small. Since the equivalence of all equilibrium constants was assumed in the derivation of the equations descriptive of the random association case, it is perhaps not surprising that the discrete association model does almost as well in reproducing the experimental data. Whether further purification of the protein to achieve enhanced homogeneity and definitive investigations of the dependence of the partial specific volume with pressure and with association can improve the situation in making the choice more definitive remains to be seen.

The results do demonstrate that association of chymotrypsinogen A in Veronal buffer solutions at pH 7.9 does take place and that the process is readily reversible. Of added interest is the fact that the actual solutions used constitute nonideal systems and that activity coefficient terms must be evaluated and applied before any definitive analysis of the data can be achieved. The exact nature of the forces involved in the association reaction remains unknown.

Acknowledgments

We are also much indebted to Messrs. D. A. Albright, J. C. Nichol, and R. C. Deonier for their advice, interest, and aid.

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Limitations Inherent in the ΔpH Method of Determining Binding Isotherms of Bovine Serum Albumin*

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ABSTRACT: Binding isotherms, determined by the ΔpH method of Scatchard and Black, of a large variety of aliphatic and aromatic anions, containing up to 14 carbon atoms, have been compared with isotherms obtained with the same substances by equilibrium dialysis and in two instances by measurement of potentials across permselective membranes. With symmetric nondeformable ions, *e.g.*, aromatic anions, the ΔpH method gives results in good agreement with those determined by dialysis. With aliphatic long-chain anions, the amount of binding and the binding constants are systematically underestimated by factors which increase with the affinity. In these comparisons, due regard has been taken of (a) the effects of the necessary differences in the amounts of competing electrolyte present when each of the three methods is used, and (b) need to correct for the lack of buffering capacity in very dilute solutions of unbuffered protein. Two methods for evaluation of an empirical electrostatic factor, ω, from the ΔpH binding measurements in 0.001 M NaCl are discussed. Explanation of the much lower ω so computed from that calculated by application of Debye–Hückel theory is sought in terms of applicability of the Linderström-Lang model to noncovalently bound long-chain ions with hydrophobic tails, and of differences between the effective charge Z of the macroion and the charge computed stoichiometrically. It is shown that as a practical matter the ΔpH method cannot be applied to any ligand of high affinity ($K > 10^5$) because of the large effect in this method of small errors in determining concentration or protein molecular weight.

The binding of small ligands to proteins has most often been measured by equilibrium dialysis, a laborious and time-consuming method which precludes any possibility of measuring amounts bound soon after mixing. If the ligand of interest is ionic in nature, *i.e.*, carries a charge, the free-ligand concentration (and hence the binding) may be estimated by electromotive force measurements with electrodes specific for the ligand (Scatchard *et al.*, 1950) or by permselective membrane techniques (Carr, 1955; Scatchard *et al.*, 1957, 1959; Baker and Saroff, 1965). These methods, where applicable, have been shown to give very reliable results. However, electrodes specifically reversible to ligands such as organic anions are rare and have been reported for only a relatively small number of simple ions, and for no highly asymmetric ones. Relatively short-time binding information may be obtained by other methods (Klotz, 1953; Steinhardt and Reynolds, 1969) such as depolarization of fluorescence (Laurence, 1952; Weber, 1952) and gel filtration (Hummel and Dreyer, 1962; Fairclough and Fruton, 1966), but they have not been used for routine and rapid analysis of a large number of protein–ligand systems.

As a part of an extensive study of the interaction of a number of proteins with a series of highly purified ligands, including long-chain anionic detergents, we have examined (a) the reliability, accuracy, and limits of applicability of the ΔpH method for measuring ion binding to bovine serum albumin; and (b) the utility and reliability of the permselective electromotive force method with large asymmetric anions (*i.e.*, octyl sulfate and dodecyl sulfate).

In 1941, Steinhardt showed that changes in position on the pH coordinate of the titration curves of wool fibers obtained with a series of strong acids reflected differences in the tendency of the protein to combine with the respective anions. In 1949, Scatchard and Black demonstrated that the binding of small inorganic anions to serum albumin could be computed from the observed shift in pH produced by addition of ligand as a neutral salt to a system buffered solely by protein. The binding of K^+ to lactoglobulin (Basch and Timasheff, 1967) and of K^+ and Na^+ to α_s-casein (Ho and Waugh, 1965) has been measured by this technique (hereafter referred to as the ΔpH method). It has also been applied in a study of the interaction of several inorganic salts with myosin (Ghosh and Mihalyi, 1952).

In addition to the long-chain ligands, we have examined binding of a number of aromatic anions by the ΔpH method. For all the ligands reported here, we include for comparison binding measurements on the same substances by equilibrium dialysis. Since Scatchard and Black (1949) achieved less satis-

* From the Department of Chemistry, Georgetown University, Washington, D. C. 20007, and the National Bureau of Standards, Washington, D. C. 20234. *Received January 28, 1969.* This work was supported by Grant GM 12085 from the U. S. Public Health Service to J. S. It includes material from the Ph.D. dissertation of J. M. C.

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