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MEMORANDUM

RM-5254-PR

APRIL 1967

PROTEIN BINDING OF SMALL IONS—
A MATHEMATICAL MODEL OF
SERUM ALBUMIN

E. C. DeLand and Rose Heirschfeldt

PREPARED FOR:

UNITED STATES AIR FORCE PROJECT RAND

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This research is supported by the United States Air Force under Project RAND, Contract No. F11620-67-C-0015, monitored by the Directorate of Operational Requirements and Development Plans, Deputy Chief of Staff, Research and Development, Hq. USAF. Views or conclusions contained in this Memorandum should not be interpreted as representing the official opinion or policy of the United States Air Force.

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PREFACE

This Memorandum reports some of the detailed background research for the eventual mathematical simulation of viable chemical systems. Because the characteristics of a viable system are determined in a large part by the chemical behavior of its proteins, a study of such a system must finally center on the chemistry of these complex molecules. Accordingly, this report is a first attempt to simulate in detail the chemical behavior of human serum albumin. Our interest lies in building an adequate model of the important proteins of blood so that the biochemistry of blood as a total system might be clarified. With that broader perspective in mind, this Memorandum should be of interest to physiologists as well as biochemists.

This research was supported in part by the Department of Health, Education, and Welfare Grant HE-08665 (National Institutes of Health).

SUMMARY

Serum albumin is an especially reactive molecule, binding both positive and negative small ions, organic molecules, and even neutral molecules. As such, its role in blood biochemistry is important and pervasive. This Memorandum examines the role of serum albumin with respect to the binding of H^+ , Cl^- and Ca^{++} .

The point of view, following much of the literature, is that the Linderstrom-Lang theory--which treats the molecule as a charged sphere--can explain the anomalous behavior of serum albumin in acidity regions remote from the isoionic point. But it is shown, first for H^+ and then for Cl^- and Ca^{++} , that while the charged sphere concept can be stretched to fit the laboratory data, the consequent Debye-Hückel parameter bears little relation to the predicted values.

The fairly exact calculations of simultaneous events possible with the computer leaves little room for doubt that a much improved theory must be developed. In fact, one conclusion is that this theory is only approximate and that an adequate explanation probably will have to begin with the exact geometry of the molecule and the

consequent intramolecular interactions. This latter procedure is almost possible today--with increasing knowledge of molecular structure--whereas it was not when the former theory was promulgated.

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I. INTRODUCTION

Study of protein reactions in a mathematical model can provide information about the protein and can supply new chemical hypotheses not as readily obtainable by standard laboratory procedures. This Memorandum presents an experimental study of serum albumin considered as a mathematical entity. The principal purposes are to show investigative techniques, and to reach certain conclusions about the protein. However, several minor objectives relate to an interest in serum albumin as a part of a larger physiological system, the blood, and the requirements for modeling that system.

Clearly, serum albumin plays an important role in blood biochemistry, but its detailed mechanisms under various conditions are not well understood. Like proteins generally, its detailed behavior is exceedingly complex. Under various conditions, serum albumin is known to bind reversibly a wide variety of small ions of either sign, and even neutral molecules. To cite only a few examples from the literature, both organic [1,2] and inorganic [3,4,5,6] anions are bound, as well as cations [7,8], hydrogen ions [9,10,11,12], and uncharged

small molecules [13,14]. This characteristic of serum albumin--plus its considerable role as a bearer of fixed (i.e., impermeable) negative charge--makes it of central interest to a model of blood chemistry and worthy of individual examination. To this purpose, a mathematical model is useful because it supplies a flexible format for ordering great detail and for interrelating the protein's various reactions.

This Memorandum considers only indirectly serum albumin's molecular structure and intramolecular properties--as they may relate to possible uncovering of binding sites at extreme acid or alkaline values. Instead, the emphasis of this feasibility study is upon the interpretation of binding data using the Linderstrom-Lang theory. The work of Tanford and Kirkwood [15], and others, suggests a more sophisticated interpretation for later work. For now, the serum albumin molecule will be considered to be a spherical mass of molecular weight 69,000 with all binding sites located on or near the surface (at least in the central range of pH). We ignore for the time being the probable cloud of oriented water molecules surrounding each charged site, except as it may effect the apparent binding energy of a site as it is

measured. Most of the experiments are performed in a 1.0 millimolar water solution in which, also, the ionic strength is maintained at 0.15 with NaCl; the temperature is 25°C. Exceptions to these data will be discussed as they arise.

II. PROCEDURE

The mathematical procedure used essentially derives from Shapiro and Shapley [16], Shapiro [17], and Clasen [18,19]. It is based upon the calculation of a chemical equilibrium by the minimization of the Gibbs' free energy function under the conditions of the experiment. That is, the concentration of each product species at equilibrium is determined from a mixture of chemical components so that the total free energy of the system is minimized. For these experiments, the temperature and pressure are constant and slight volume changes of the single phase may be ignored. The method follows that of Gibbs [20,21], using Lagrange multipliers. This procedure is used (rather than either the simultaneous solution of mass-action equations [22] or the kinetic differential equations [23]) out of consideration for the numerical techniques on the computer, and because the kinetic rate constants generally are not available. We are thus concerned only with systems at equilibrium; but for conditions where a slow denaturation may occur, e.g., at high pH, time delays and a differential trajectory may be simulated.

Reference 16 shows conditions for the existence of a unique solution for the equilibrium problem; Ref. 17 gives

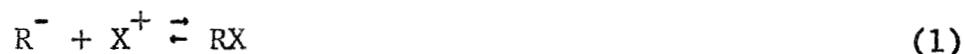
a method--the method of "conceptual compartments"--upon which we depend heavily, for considerably reducing the numerical size (number of equations) to be considered. References 18 and 19 discuss the generalized equations for computer application, and a method for solution. As required, these results will be subsequently elaborated.

The mathematical procedure has been applied previously in analyses of other systems; e.g., preliminary analyses of blood in De Haven and DeLand [24], and DeLand [25]. In practice, a complete list of the pertinent reversible chemical reactions is required, along with the equilibrium constant for each reaction at the given temperature and pressure. In addition, the moles of each input component are required, as well as the valence of each species for the conservation of mass and charge equations. Always, if only implicitly, a condition of zero net electrical charge (considering the valence of each species) is imposed.

Characteristically, as in the present case, not all of the required data are known; and various curve-fitting and data-point-fitting techniques have been incorporated for determining unknown parameters. For example, the essence of the present problem is to determine apparent

dissociation constants to fit empirical data and, if possible, to rationalize the relations between "apparent" and "intrinsic" constants for serum albumin.

By an intrinsic constant we will always mean the reaction constant K_i given in very dilute solutions for simple reactions of the type



by mass-action equations of the form

$$c_R c_X = K_i c_{RX} \quad (2)$$

where the symbol c_X indicates concentration of the substance X in appropriate units, and K_i is the intrinsic dissociation constant for the reaction.

By the effective dissociation constant for reaction (1) will be meant a modified intrinsic constant resulting from a variety of sources; e.g., a) the electrostatic charge effects arising from the Linderstrom-Lang theory, b) competition for binding sites by various ligands, c) "salt-bridging" between positive and negative sites on the protein [9], d) hydration of the molecule, which may

materially change the characteristics of the molecule [26]. Thus, in place of Eq. (2), at any concentration the effective K will be given by

$$\hat{x} \hat{r} = K_e \hat{xr} \quad (3)$$

where the symbol \hat{x} indicates the activity [27] (effective concentration) of the species X . If γ_x is the activity coefficient, we may also write

$$(\gamma_X c_X) (\gamma_R c_R) = K_e (\gamma_{XR} c_{XR})$$

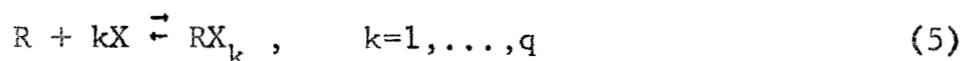
where c_X is the concentration and the activity coefficients vary with concentration in such a way that K_e remains constant (a function only of temperature and pressure).

In practice, the activity coefficients are not frequently determined since this requires a knowledge of both c_X and \hat{x} . More often, either c_X is known quantitatively or \hat{x} is measurable, e.g., by use of an instrument which measures the apparent activity of an ion. This impasse leads to the definition of an "apparent" dissociation constant, K_a , given by

$$c_R c_X = K_a c_{RX} \quad (4)$$

where, as in the computer model, the concentrations c_X are known quantitatively, but K_a is a function of the particular experimental conditions, e.g., ionic strength or molecular geometry. For serum albumin there has been considerable discussion of the apparent reaction constants-- particularly, the hydrogen ion titration curve--and we continue that discussion here.

Finally, for a macromolecule R having q sites for combination with a ligand X according to one of the q reactions of the form



there are q apparent constants defined by

$$c_R c_X^k = K_k c_{RX_k}, \quad k \leq q \quad (6)$$

where RX_k is a representative molecule having exactly k arbitrarily chosen sites occupied. K_k may be assumed not to vary with the pattern of ionization (which k of the q sites are occupied), which is equivalent to requiring that the q sites be independent, equivalent, and without interaction [28]. When this is the case, as a first approximation

K_k may be assumed to be a simple function of k (as in Ref. 12, p. 493), i.e.,

$$K_k = \frac{q - k + 1}{k} K_1 . \quad (7)$$

But since the total charge on a protein is a function of k , and interaction generally may not be discounted, this assumption is usually not tenable for a protein (as will be shown in the next section).

III. DATA

It may be assumed that human serum albumin (HSA) is very similar to bovine (BSA) [9], with which most empirical experiments have been made, and that its reactions are stable and reversible in the pH region 4.3 to 10.5 [9]. Outside of this region, the molecule is also well-behaved in the sense that its reactions and probable changes in structure are reversible and reasonably (10 minutes) time independent. BSA is formally a well-known molecule; we take as its descriptors the data of Tanford, et al. [9]. Rational deviations in the extrinsic data--e.g., following Tanford [9], using 69,000 for the molecular weight instead of 65,000--make only an expected proportionate difference in the results (as will be shown in Sec. IV). But generally, Ref. 9--henceforth simply "Tanford"--supplies the "standard data" for the mathematical model. Obviously, any of the data in this model could be changed, with reason, without changing the procedure--a facility that is experimentally useful.

The distinction between components and species will not precisely be that of some previous work (Scatchard [29]); Casassa, et al. [30]). It is convenient to refer to the

chemical constituents put into the system as components and to the products of reactions or the substances present at equilibrium in a particular phase as species. The components need not be "elementary" or indivisible, but convenient groupings. Given the amounts of the components in moles, a computer solution will consist of the moles and mole fractions of each species at equilibrium under various conditions. Concentration units will conveniently be in mole fractions, although other units may be computed (e.g., moles per kilogram of solute, moles per liter of plasma, etc.) as required.

With respect to the ligand H^+ , the empirical data for serum albumin are shown in Table I (as summarized by Tanford) for ionic strength 0.15 and molecular weight 65,000. For molecular weight 69,000 [31], the number of titratable groups is multiplied by 69/65 even though the result may not be integral. The parenthetical numbers are within the probable uncertainty of the data, but are used in these results because of their likelihood of occurrence. There are five principal classes, therefore, and three minor classes for H^+ ionization--a total of 215 sites--in the side chains of the polypeptide. The empirical titration curve at ionic strength 0.15 is reproduced in Fig. 1. This figure also

Table I

SITES PER 65,000 GRAMS BSA

Group Class	Titrateable Groups Found	Intrinsic pK_i Observed
α -carboxyl	(1)	3.75
β -carboxyl	99	4.02
Imidazole	16	6.9
α -Amino	(1)	7.75
ϵ -Amino	57	9.8
Phenolic	19	10.35
Guanidine	22	12.+
Sulfhydryl	(0)	-

shows the "intrinsic" titration curve--that curve generated by computation using exactly the intrinsic pK_i and number of sites of Table I. It remains to modify the intrinsic constants in a rational way to fit the empirical curve.

The components (inputs) of the mathematical system for the "intrinsic" curve of Fig. 1 are 1.0 mM HSA, 150 mM NaCl, and 55.3436 moles H_2O (1.0 L at 25°C). The volume including protein is therefore approximately 1146.3 cc, using the molar volume of protein as given for human plasma [32]. The data computed for the curve in Fig. 1 are the moles and mole-fractions of the species H^+ , OH^- , H_2O , Na^+ , Cl^- , HSA, and the list of all ionizing classes

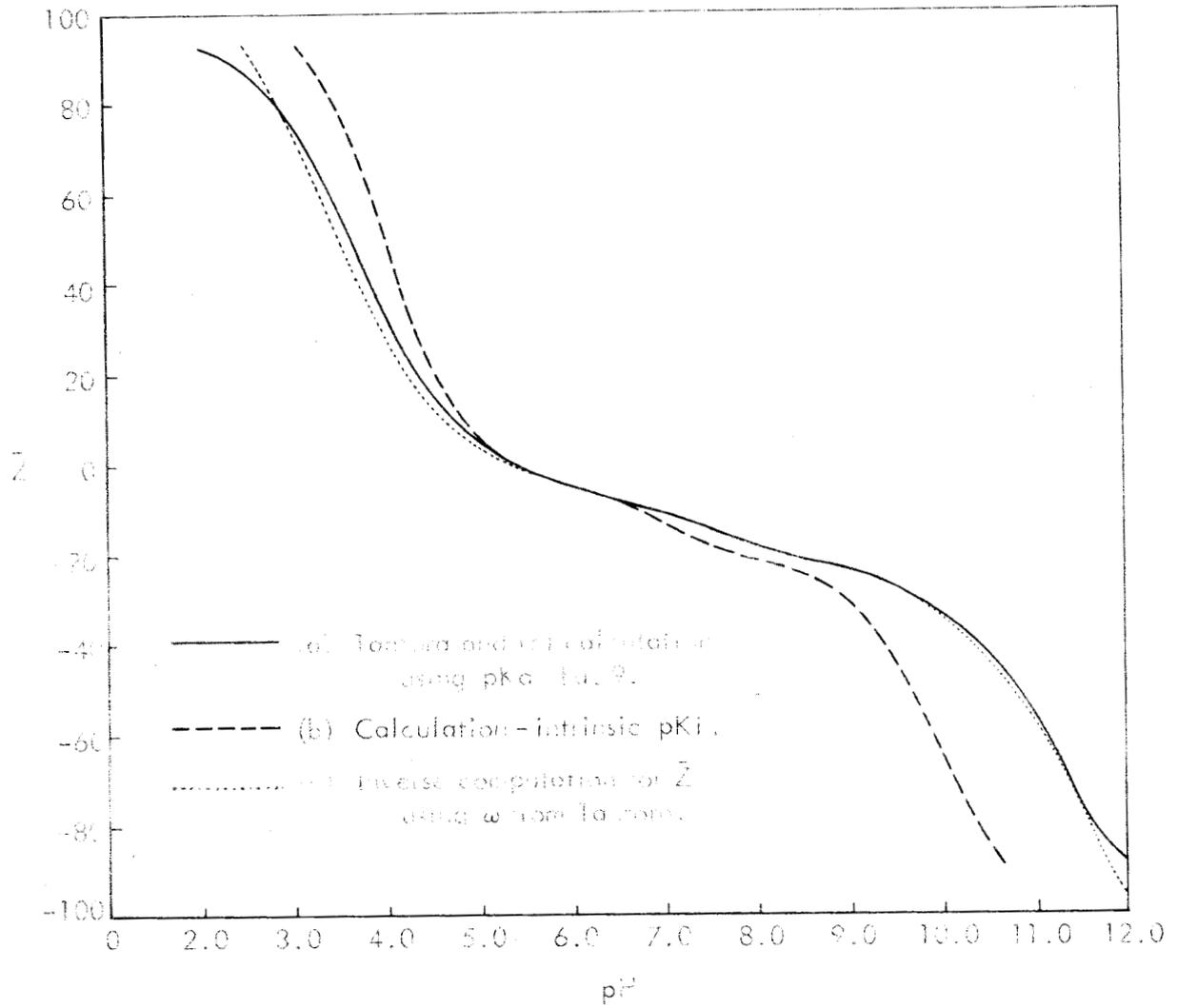


Fig. 1--Hydrogen ion titration of BSA at 25°C.,
ionic strength 0.15

showing the fraction ionized at each pH. Table II, for example, is a reproduction of the computer results for pH 5.5 and pH 4.5 using the intrinsic pK_1 .

In Table II, the mole numbers of each species may also be interpreted as moles per liter of water since just one liter of the solvent is present. The mole-fraction numbers for the species H^+ , OH^- , H_2O , Na^+ , Cl^- , and HSA sum to one; i.e., the moles of ionizing species are not, of course, counted in the total moles of solution. The ionizing sites are listed in a separate column of the table indicating that the ratio ionized and the moles of H^+ released to the solution are computed separately.*

With no Cl^- or other small ion binding except H^+ for the first experiments, it will be assumed that \bar{Z} , the net

*More precisely, "computed separately" means: We assume the sites within each class to be equivalent and independent. Then, as in Eqs. (5) and (6), we may regard each class of q sites as equivalent to q monobasic acids--each at the concentration of the protein and each having the ionization constant K_a , the average apparent constant for that class [12]. Following Shapiro [17], the ionization of each class is then computed in a conceptual sub-compartment of the protein solution. The restriction regarding the independence of the sites does not preclude the Linderstrom-Lang theory, which deals with molecular, macroscopic charge variation.

Table II

Computed distribution of species for curve b, Fig. 1

a) pH 5.35				b) pH 4.45			
X-BAR		5.56446E 01	2.28320E-01	X-BAR		5.56326E 01	2.28320E-01
PH		5.34874E 00	-0.	PH		4.45554E 00	-0.
H+	MOLES	4.47971E-06	-0.	H+	MOLES	3.50233E-05	-0.
	MFRAC	8.05057E-08	-0.		MFRAC	6.29546E-07	-0.
OH-	MOLES	2.26505E-09	-0.	OH-	MOLES	2.89589E-10	-0.
	MFRAC	4.07056E-11	-0.		MFRAC	5.20538E-12	-0.
H2O	MOLES	5.53436E 01	-0.	H2O	MOLES	5.53316E 01	-0.
	MFRAC	9.94591E-01	-0.		MFRAC	9.94589E-01	-0.
NA+	MOLES	1.50000E-01	-0.	NA+	MOLES	1.38000E-01	-0.
	MFRAC	2.69568E-03	-0.		MFRAC	2.48056E-03	-0.
CL-	MOLES	1.50000E-01	-0.	CL-	MOLES	1.62000E-01	-0.
	MFRAC	2.69568E-03	-0.		MFRAC	2.91196E-03	-0.
PROTN	MOLES	9.99990E-04	-0.	PROTN	MOLES	9.99989E-04	-0.
	MFRAC	1.79710E-05	-0.		MFRAC	1.79749E-05	-0.
ACOOH	MOLES	-0.	2.59095E-05	ACOOH	MOLES	-0.	1.73661E-04
	MFRAC	-0.	1.13479E-04		MFRAC	-0.	7.60602E-04
ACOO-	MOLES	-0.	1.03409E-03	ACOO-	MOLES	-0.	8.86339E-04
	MFRAC	-0.	4.52913E-03		MFRAC	-0.	3.88200E-03
BCOOH	MOLES	-0.	4.68490E-03	BCOOH	MOLES	-0.	2.80946E-02
	MFRAC	-0.	2.05190E-02		MFRAC	-0.	1.23049E-01
BCOO-	MOLES	-0.	1.00415E-01	BCOO-	MOLES	-0.	7.70054E-02
	MFRAC	-0.	4.39800E-01		MFRAC	-0.	3.37269E-01
IMID+	MOLES	-0.	1.65329E-02	IMID+	MOLES	-0.	1.69388E-02
	MFRAC	-0.	7.24109E-02		MFRAC	-0.	7.41888E-02
IMID	MOLES	-0.	4.67133E-04	IMID	MOLES	-0.	6.12032E-05
	MFRAC	-0.	2.04596E-03		MFRAC	-0.	2.68059E-04
AMIN+	MOLES	-0.	1.05579E-03	AMIN+	MOLES	-0.	1.05946E-03
	MFRAC	-0.	4.62415E-03		MFRAC	-0.	4.64024E-03
AMIN	MOLES	-0.	4.21373E-06	AMIN	MOLES	-0.	5.40722E-07
	MFRAC	-0.	1.84554E-05		MFRAC	-0.	2.36827E-06
EAMIN+	MOLES	-0.	6.04979E-02	EAMIN+	MOLES	-0.	6.04997E-02
	MFRAC	-0.	2.64970E-01		MFRAC	-0.	2.64978E-01
EAMIN	MOLES	-0.	2.15192E-06	EAMIN	MOLES	-0.	2.75194E-07
	MFRAC	-0.	9.42502E-06		MFRAC	-0.	1.20530E-06
PHENO	MOLES	-0.	2.01998E-02	PHENO	MOLES	-0.	2.02000E-02
	MFRAC	-0.	8.84714E-02		MFRAC	-0.	8.84722E-02
PHENO-	MOLES	-0.	2.02502E-07	PHENO-	MOLES	-0.	2.58960E-08
	MFRAC	-0.	8.86923E-07		MFRAC	-0.	1.13420E-07
GUAN+	MOLES	-0.	2.34000E-02	GUAN+	MOLES	-0.	2.34000E-02
	MFRAC	-0.	1.02488E-01		MFRAC	-0.	1.02488E-01
GUAN	MOLES	-0.	1.66071E-09	GUAN	MOLES	-0.	2.12370E-10
	MFRAC	-0.	7.27360E-09		MFRAC	-0.	9.30141E-10

molecular charge, and ΔH^+ (counting from the isoionic point) are equal. The isoionic point is taken as pH 5.35 when the molecule has zero net charge, i.e.,

$$\bar{Z} = -x_{\text{COO}^-} + x_{\text{IMID}^+} + x_{\text{eNH}_3^+} - x_{\text{RO}^-} + x_{\text{GNH}_3^+} = 0 \quad (8)$$

where x_j are the moles of ionized sites in the five principal classes. The computed titration curve (see Fig. 1b) begins at pH 5.35, and proceeds by simultaneous addition of HCl and subtraction of NaOH, maintaining constant ionic strength, so that $\bar{Z} = \Delta H = \Delta \text{Cl}^- - \Delta \text{Na}^+$.

That the actual isoionic point with the intrinsic pK_i is pH = 5.35 can be seen in Table IIIA, where by addition Eq. (8) is satisfied and, for example, for the imidazole class

$$\text{pK}_i = 6.9 = -\log_{10} \frac{(2.0715 \times 10^{-3} \times 8.019 \times 10^{-8})}{(7.3083 \times 10^{-2} \times 55.3436)} \quad (9)$$

In Eq. (9) the concentration numbers are taken directly from the equilibrium solution in mole-fractions so that 55.3436 moles/liter of water at 25°C is a conversion factor to pK_i in moles per liter of H_2O . Equation (9) holds for each species at equilibrium for any pH.

Table III

Computed distribution of species for:

a) The isoionic point using
intrinsic pK_i

b) The isoionic point using
reduced volume of solvent
(1.0 liter of solution)

X-BAR	5.56446E-01	2.28320E-01	K-BAR	4.75478E-01	2.28320E-01
PH	5.35324E-00	-0.	PH	5.35276E-00	-0.
H+	MOLES 4.44594E-06	-0.	H+	MOLES 3.79968E-06	-0.
	MFRAC 7.98988E-08	-0.		MFRAC 7.99128E-08	-0.
OH-	MOLES 2.28225E-09	-0.	OH-	MOLES 1.94802E-09	-0.
	MFRAC 4.10148E-11	-0.		MFRAC 4.09696E-11	-0.
H2O	MOLES 5.53436E-01	-0.	H2O	MOLES 4.72468E-01	-0.
	MFRAC 9.94591E-01	-0.		MFRAC 9.93669E-01	-0.
NA+	MOLES 1.50000E-01	-0.	NA+	MOLES 1.50000E-01	-0.
	MFRAC 2.69568E-03	-0.		MFRAC 3.15472E-03	-0.
CL-	MOLES 1.50000E-01	-0.	CL-	MOLES 1.50000E-01	-0.
	MFRAC 2.69568E-03	-0.		MFRAC 3.15472E-03	-0.
PROTN	MOLES 1.00001E-03	-0.	PROTN	MOLES 9.99995E-04	-0.
	MFRAC 1.79714E-05	-0.		MFRAC 2.10313E-05	-0.
ACOOH	MOLES -0.	2.57189E-05	ACOOH	MOLES -0.	2.57233E-05
	MFRAC -0.	1.12644E-04		MFRAC -0.	1.12663E-04
ACOO-	MOLES -0.	1.03428E-03	ACOO-	MOLES -0.	1.03428E-03
	MFRAC -0.	4.52996E-03		MFRAC -0.	4.52994E-03
BCOOH	MOLES -0.	4.65115E-03	BCOOH	MOLES -0.	4.65193E-03
	MFRAC -0.	2.03712E-02		MFRAC -0.	2.03746E-02
BCOO-	MOLES -0.	1.00449E-01	BCOO-	MOLES -0.	1.00448E-01
	MFRAC -0.	4.39948E-01		MFRAC -0.	4.39944E-01
IMID+	MOLES -0.	1.65294E-02	IMID+	MOLES -0.	1.65295E-02
	MFRAC -0.	7.23958E-02		MFRAC -0.	7.23962E-02
IMID	MOLES -0.	4.70583E-04	IMID	MOLES -0.	4.70503E-04
	MFRAC -0.	2.06107E-03		MFRAC -0.	2.06072E-03
AMIN+	MOLES -0.	1.05575E-03	AMIN+	MOLES -0.	1.05576E-03
	MFRAC -0.	4.62401E-03		MFRAC -0.	4.62401E-03
AMIN	MOLES -0.	4.24561E-06	AMIN	MOLES -0.	4.24487E-06
	MFRAC -0.	1.85950E-05		MFRAC -0.	1.85918E-05
EAMIN+	MOLES -0.	6.04978E-02	EAMIN+	MOLES -0.	6.04979E-02
	MFRAC -0.	2.64969E-01		MFRAC -0.	2.64970E-01
EAMIN	MOLES -0.	2.16827E-06	EAMIN	MOLES -0.	2.16789E-06
	MFRAC -0.	9.49661E-06		MFRAC -0.	9.49495E-06
PHENO	MOLES -0.	2.01998E-02	PHENO	MOLES -0.	2.01998E-02
	MFRAC -0.	8.84714E-02		MFRAC -0.	8.84714E-02
PHENO-	MOLES -0.	2.04040E-07	PHENO-	MOLES -0.	2.04005E-07
	MFRAC -0.	8.93660E-07		MFRAC -0.	8.93503E-07
GUAN+	MOLES -0.	2.34000E-02	GUAN+	MOLES -0.	2.34000E-02
	MFRAC -0.	1.02488E-01		MFRAC -0.	1.02488E-01
GUAN	MOLES -0.	1.67332E-09	GUAN	MOLES -0.	1.67303E-09
	MFRAC -0.	7.32885E-09		MFRAC -0.	7.32757E-09

To test the effect of a more concentrated solution on the isoionic point and subsequent points on the curve, the moles of water were reduced to $47.2468 = 853.7$ ml 25°C , which with 146.3 ml of protein makes just one liter of solution. Table IIIB shows the distribution of species at the isoionic point for comparison with Table IIIA.

IV. RESULTS

A. LINDERSTROM-LANG EFFECTS

Introduction of the Linderstrom-Lang effects on the pK_i is an iterative procedure on the computer. It is required to find $\omega = \omega(\text{pH})$ so that a set of apparent constants pK_a related to the pK_i by

$$(pK_a)_j = \text{pH} - \log \frac{x_j}{1 - x_j} = (pK_i)_j - 0.868 \omega \bar{Z} \quad (10)$$

will yield the empirical titration curve. In Eq. (10), x_j is the fractional dissociation of sites in class j at the given pH; the $(pK_a)_j$ are modifications of the $(pK_i)_j$ owing to the net charge on the spherical molecule of HSA. The parameter ω and the pK_a are functions of pH; the former also varying with the effective radius of the molecule, the dielectric constant of the milieu, the ionic strength, competition of other ligands with H^+ , and the temperature. In practice, ω is simply an empirical parameter. \bar{Z} , the net protein charge, varies with ΔH^+ ; but also with minor ligand binding, e.g., Cl^- , and in this circumstance, $\Delta\text{H}^+ \neq \bar{Z}$.

In computation, there is a choice of iterative procedures to find ω . Beginning at the isoionic point, one may titrate to, say, pH 4.5 using the pK_i (obtaining the dashed curve of Fig. 1), and then at constant pH vary ω until $\bar{Z} = \bar{Z}(\text{pH } 4.5)$ lies on the desired curve. Alternately, beginning at the isoionic point, one may increment ΔH^+ (adding and subtracting HCl and NaOH) to $\bar{Z}(\text{pH } 4.5)$, and then vary ω until pH = 4.5. In either case, ending on the empirical curve at the same point must yield the same value of ω . (One may also invert the procedure, given ω , and solve for \bar{Z} at each point, Fig. 1c.)

It is assumed that as the charge on the spherical protein molecule changes, all sites are equally effected and not just those yet un-ionized. That is, all pK_a values are a function of \bar{Z} regardless of the class of group or site position within the class. Refinements regarding the electrostatic interaction factors within each class are now under investigation--especially, the surface or interior location of these sites and possible existence of salt-bridges between oppositely charged sites.

Figure 1d (indistinguishable from Fig. 1a) is a plot of the titration curve generated using Eq. (10), and Fig. 2b is a plot of the corresponding $\omega(\text{pH})$. Figure 2a, for

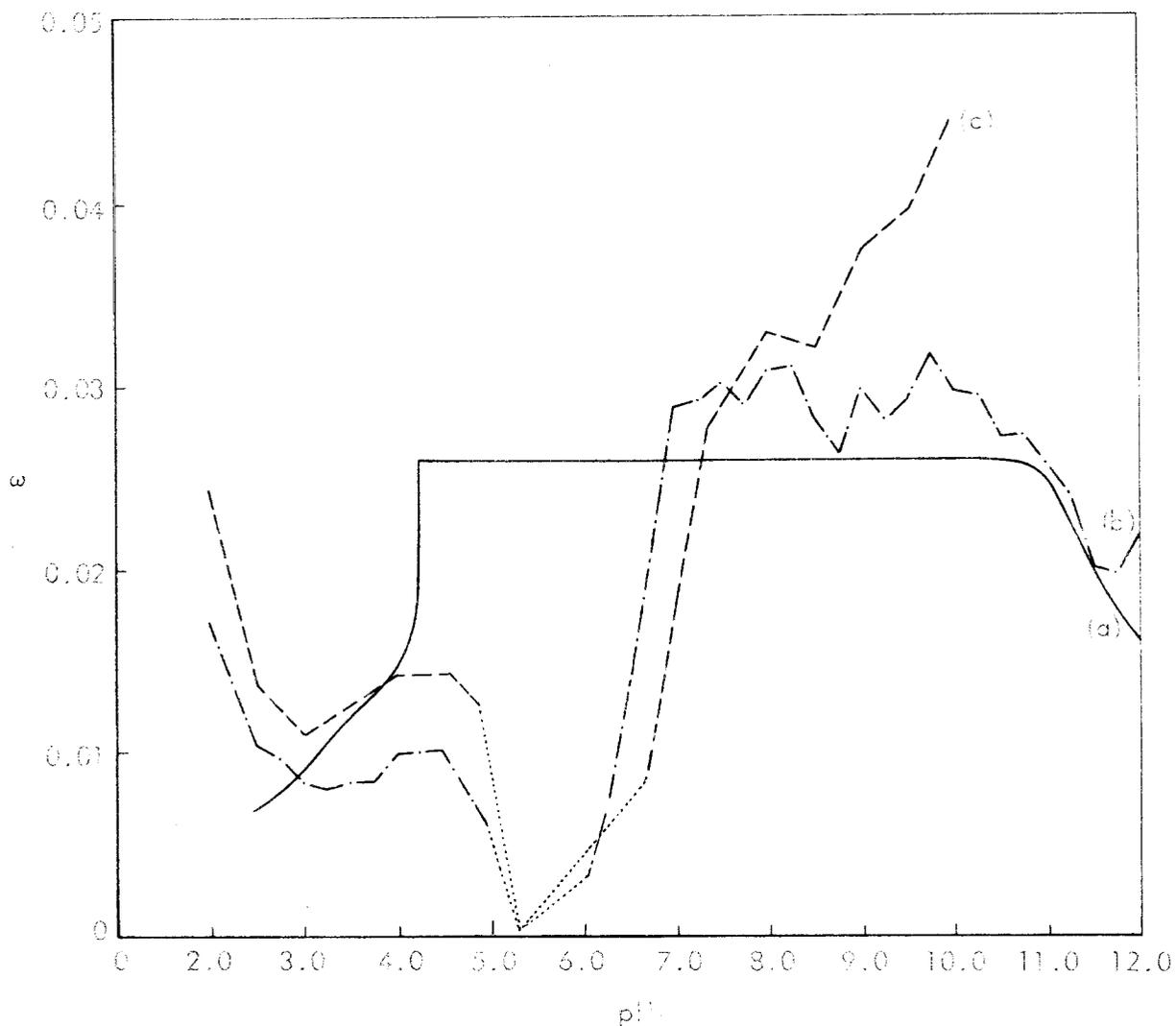


Fig. 2--The Debye-Hückel empirical parameter, ω , as a function of pH:
a) Derived from the laboratory data, Tanford et al.,
b) Calculated using isoelectric point 5.35 and no competitive binding (cf. Fig. 1c),
c) Calculated with Cl^- binding (acidic pH), and with Ca^{++} binding (alkaline pH)

convenience, is the plot of ω from Tanford, where Cl^- binding was also considered.

A significant aspect of this computation for ω is the assumption that ω is a continuous function of pH, particularly for small values of \bar{Z} . Because at $\bar{Z} = 0$, ω is indeterminate (Eq. (10)), a first attempt was made to determine ω at $\bar{Z} = 0$ by a limit process. But for values near the isoionic point \bar{Z} cannot be determined with sufficient accuracy, and an analytic approximation to \bar{Z} was deemed insupportable on the data available. That $\omega = 0$ at $\bar{Z} = 0$ may be argued, however, from the fact that the isoionic point is an arbitrary reference point. If, instead, the extreme acid end of the curve is taken as a reference with the parameter $\bar{h} = 96 - \bar{Z}$, then Eq. (10) becomes

$$(\text{pK}_a)_j = (\text{pK}_i)_j - 0.868 \bar{\omega} \bar{h} ; \quad (11)$$

and $\bar{\omega}$ must go to zero at pH 5.35 since at the isoionic point $\text{pK}_a = \text{pK}_i$ for all j , but $\bar{h} \neq 0$.

Accordingly, the plot of ω in the central range is extrapolated through zero with dotted lines forming, at the same time, a continuous function of pH. The sensitivity

of the value of ω with \bar{Z} varies with the absolute value of \bar{Z} , being very insensitive when \bar{Z} is large. Figure 2a is a plot of the computed ω from Eq. (11). In a similar vein, Fig. 1c is the inverse computation for \bar{Z} using Eq. (10): assuming $\omega = 0.026$ in the central range of pH find the corresponding \bar{Z} .

B. AN EFFECTIVE TITRATION CURVE

Because of a requirement for other applications [25], and because of the insight afforded, it was instructive to develop an intermediate, heuristic titration curve for HSA. This "effective" curve was required to "fit" the Tanford curve for BSA as closely as possible by adjusting the available parameters once-and-for-all, i.e., independent of pH. Essentially, there are but three sets of parameters available to vary the effective fit: a) the pK_a for each class of sites, b) the number of sites per class, and c) the pK_a for individual sites within a class.

Figure 3b shows the effective titration curve obtained using the Tanford data except for adjusting only the pK_a for each class. The pK_a 's required are COOH = 3.80, Imidazole = 7.20, Amino = 8.0, Phenolic = 10.7, and Guanidine = 12.0+. That is, if the several sites were

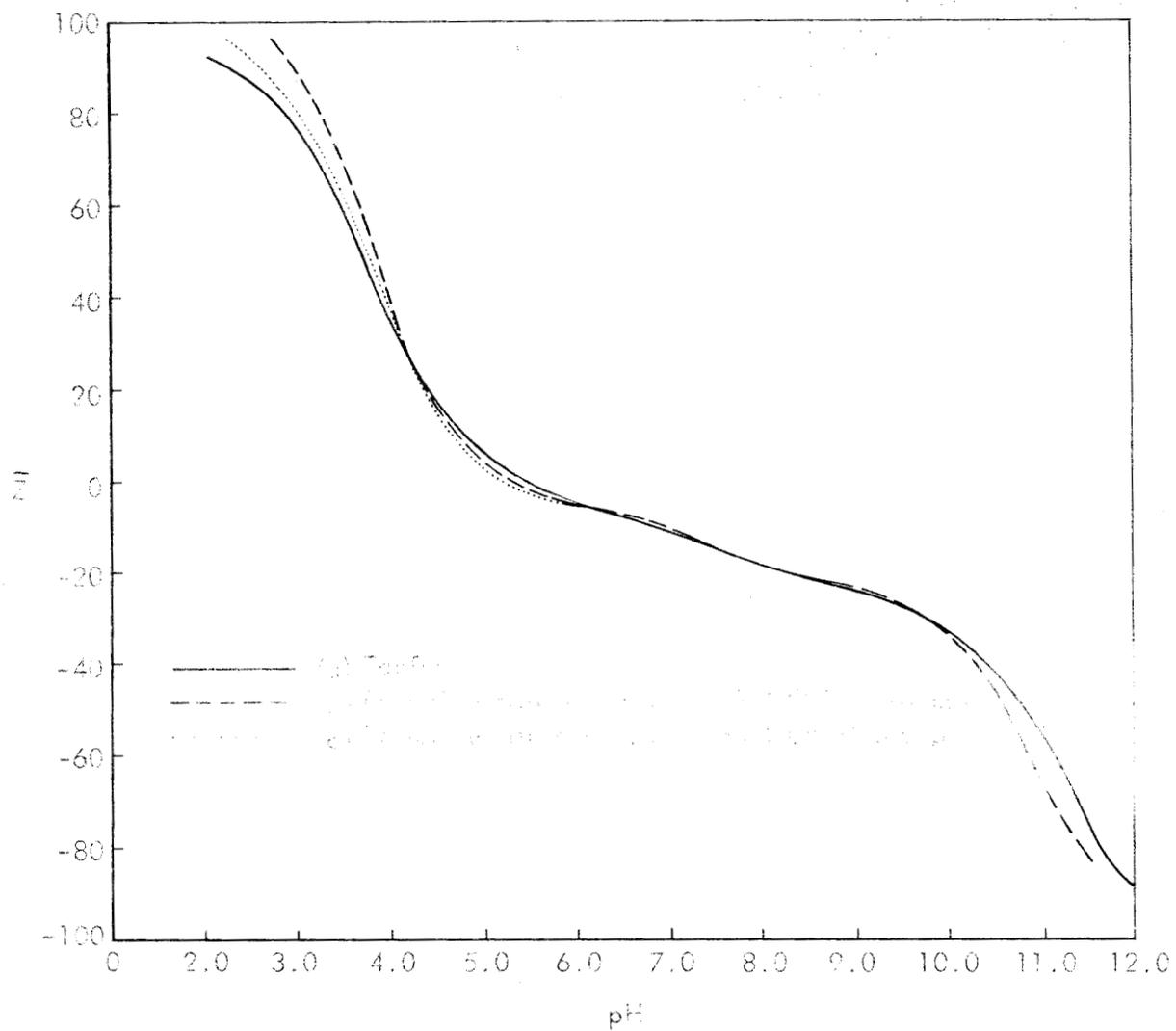


Fig. 3--Computed titration of BSA at 25°C.,
ionic strength 0.15

completely independent, electrostatic effects were inoperative, and the effective pK's for each class were those given, the titration curve would look like Fig. 3b. For many applications (e.g., [25]), this effective curve is near enough; i.e., the buffering power of the protein in the physiologic pH range is correct, and the net charge on the average molecule is always within one or two H^+ units of the correct number.

A better effective fit may be obtained from the previous fit by adjusting the number of available sites per class. As an example, assume there are but 90 COOH sites rather than 99. As the titration proceeds toward the acid end, nine fewer H^+ units are buffered and the slope of the titration curve in Fig. 3c decreases along the dotted line. An implication here is that these nine sites are, in vitro, either occupied or masked by competitive small ions, or ancillary binding may significantly change \bar{Z} . The binding of Cl^- , and Ca^{++} (to be examined next) can account for these discrepancies. A similar explanation may apply at the extreme alkaline end.

A better effective fit may then be obtained by considering the pK_a of individual sites, but this leads directly to Linderstrom-Lang theory as above, or a modification thereof [33] which we do not consider here.

C. CHLORIDE BINDING

The principal references on chloride binding used for the work in this section are Saroff and Healy [4] and Scatchard, et al. [6], where, with Cl^- concentration of 0.046 in NaCl and HCl solution, Cl^- binding was obtained as shown in Fig. 4. Generally, Cl^- is bound by $-\text{NH}_3^+$ groups according to the association equation

$$k = \frac{(-\text{NH}_3^+ \text{Cl}^-)}{(-\text{NH}_3^+)(\text{Cl}^-)} ; \quad (12)$$

but evidently not all $-\text{NH}_3^+$ groups are identical in binding power since if they were a single exponential curve would result--as shown in Fig. 4b where the calculation is made with the Linderstrom-Lang equation (Eq. (9)). Both Refs. 4 and 6 suggest that the $-\text{NH}_3^+$ groups may be subdivided to explain curve 4a. We show here that this is generally true, and compute the Linderstrom-Lang constant with Cl^- binding.

The components for this model are altered to 0.046 mmoles of NaCl, which remains constant, and 0.104 mmoles Na Lactate so that the ionic strength is still 0.150 or 0.301 osmoles with the protein. The isoelectric point and the isoionic point are no longer identical. With approximately

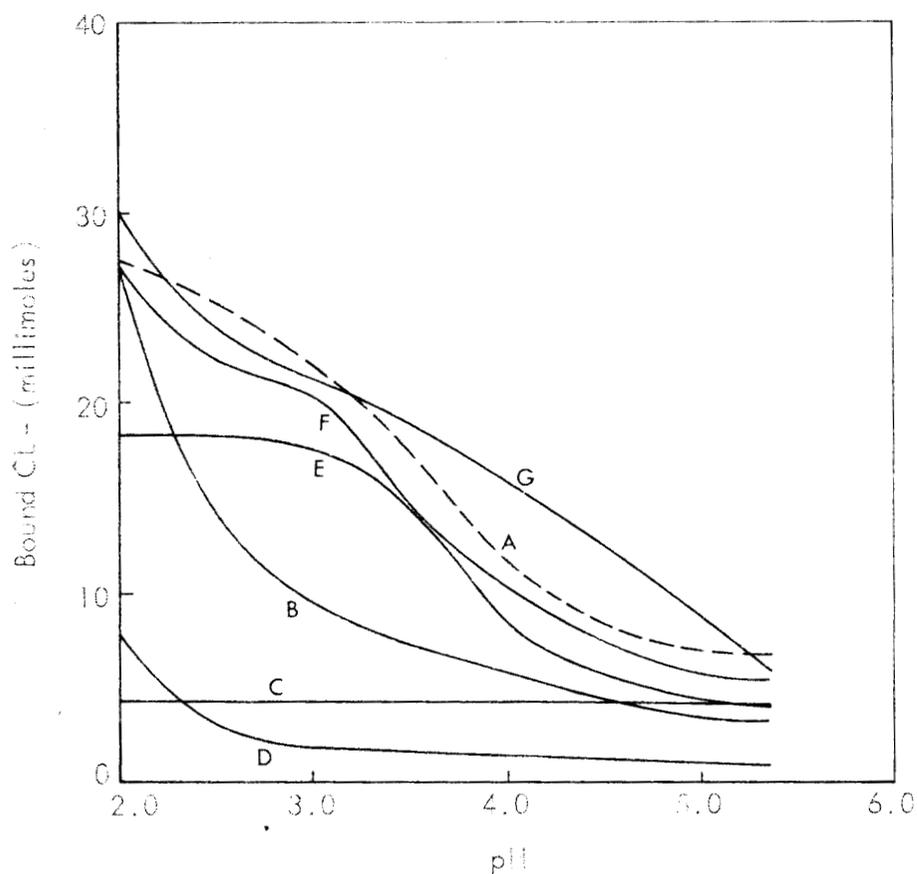


Fig. 4--Cl⁻ binding from salt-acid mixtures with Cl⁻ conc. = 0.046:

- a) Scatchard, et al.,
- b) All NH₃⁺ groups equivalent,
- c) Saturation of four NH₃⁺ sites with $k = 2500$,
- d) Binding of 84 sites with $k = 1$,
- e) 12 sites with variable ω ,
- f) Sum of all three subgroups,
- g) 12 sites with $\bar{\omega} = 4\omega$

7.0 mmoles of Cl^- bound per mmole of protein in the vicinity of pH 5.35, the total positive charges per molecule are reduced by the same amount, and the solution must be titrated into the acid range to find the point of zero charge. The isoelectric point, taking account of Eq. (9), is pH 5.15. As the titration proceeds, the Cl^- bound varies (with pH) so that an iterative procedure is required. At pH 5.15, 8.51 moles of Cl^- are bound per mole of HSA. For this computation $\omega = 0.026$.

On the other hand, the isoionic point moves to the alkaline side. For this point, the computation begins at pH 5.35 using the pK_1 and no Cl^- binding. First, Cl^- is bound to the appropriate sites, to be described, but electrostatic effects are ignored. The pH moves from 5.352 to 5.358 owing to the enhanced binding of H^+ in turn due to the reduced $-\text{NH}_3^+$ concentration. Then, with Cl^- binding, and using the electrostatic effects, Eq. (9), but with no added increment of H^+ or OH^- , the pH moves to 5.45, the new isoionic point. Of course, to call this the "isoionic" point is rather a contradiction of terms, but the meaning is clear: beginning with a true isoionic solution at pH 5.35, NaCl is added and the pH moves to 5.45 because of a shift in the effective $\text{pK}'\text{s}$.*

*This definition follows Ref. 12, p. 604.

Tables IVA and B illustrate these two computations with all $-\text{NH}_3^+$ groups equivalent. The binding constant required to produce 7.0 groups bound at pH 5.35 is $k = 2.735$, using the pK_i for H^+ binding. This compares reasonably well with the data of Saroff [4] where, to fit the curve in Fig. 4a by hand calculation, he found it necessary to assign $k = 1$ to 84 groups, $k = 50$ to 12 groups, and $k = 2500$ to 4 groups. But with $k = 2.735$ for all (approximately) 100 groups, the binding curve, even using Eq. (9), results in the single exponential curve of Fig. 4b--in particular, the binding at pH 3.0 has considerable error. Accordingly, a calculation was made using the Saroff [4] subdivision of the $-\text{NH}_3^+$ groups.

Figure 4a probably is composed of three subgroups of Cl^- binding sites which can be extracted as follows. First, considering Eq. (12) and Table IV, it is apparent that the Cl^- binding curve is generated by varying the association constant k . Practically all of the $-\text{NH}_2$ groups are in the $-\text{NH}_3^+$ form at the isoionic point so that no new sites become available for binding on the acid side. Next, when the assigned subgroups of sites are saturated with Cl^- , practically no more Cl^- can be bound regardless of how large k becomes. Now, the four groups with $k = 2500$ are

Table IV

Isoelectric and isoionic points with Cl^- binding, all NH_3^+ groups equivalent, $k = 2.735$, and using Eq. (9), $\omega = 0.026$

a) Isoelectric point = 5.15

X-BAR		5.56400E 01	2.28320E-01
PH		5.15226E 00	-0.
H+	MOLES	7.06227E-06	-0.
	MFRAC	1.26928E-07	-0.
OH-	MOLES	1.43663E-09	-0.
	MFRAC	2.58202E-11	-0.
H2O	MOLFS	5.53436E 01	-0.
	MFRAC	9.94673E-01	-0.
NA+	MOLES	1.50000E-01	-0.
	MFRAC	2.69590E-03	-0.
LACTIC	MOLES	1.04000E-01	-0.
	MFRAC	1.86916E-03	-0.
CL-	MOLES	4.13841E-02	-0.
	MFRAC	7.43784E-04	-0.
PROTN	MOLES	1.00001E-03	-0.
	MFRAC	1.79729E-05	-0.
ACOOH	MOLES	-0.	5.06714E-05
	MFRAC	-0.	2.21931E-04
ACOO-	MOLES	-0.	1.00933E-03
	MFRAC	-0.	4.42068E-03
BCOOH	MOLES	-0.	8.98508E-03
	MFRAC	-0.	3.93530E-02
BCOO-	MOLES	-0.	9.61149E-02
	MFRAC	-0.	4.20966E-01
IMID+	MOLES	-0.	1.67820E-02
	MFRAC	-0.	7.35023E-02
IMID	MOLES	-0.	2.17968E-04
	MFRAC	-0.	9.54662E-04
AMIN+	MOLES	-0.	1.05806E-03
	MFRAC	-0.	4.63410E-03
AMIN	MOLES	-0.	1.94114E-06
	MFRAC	-0.	8.50186E-06
EAMIN+	MOLES	-0.	6.04990E-02
	MFRAC	-0.	2.64975E-01
EAMIN	MOLES	-0.	9.89217E-07
	MFRAC	-0.	4.33259E-06
PHENO	MOLES	-0.	2.01999E-02
	MFRAC	-0.	8.84719E-02
PHENO-	MOLES	-0.	1.01065E-07
	MFRAC	-0.	4.42648E-07
GUAN+	MOLES	-0.	2.34000E-02
	MFRAC	-0.	1.02488E-01
GUAN	MOLES	-0.	7.63397E-10
	MFRAC	-0.	3.34354E-09
NH3+CL	MOLES	-0.	-0.4.61588-03
	MFRAC	-0.	-0.2.021 -02
NH3+	MOLES	-0.	-0.1.01739-01
	MFRAC	-0.	-0.1.000 -00

b) Isoionic point = 5.45

X-BAR		5.56400E 01	2.28320E-01
PH		5.45601E 00	-0.
H+	MOLES	3.50906E-06	-0.
	MFRAC	6.30672E-08	-0.
OH-	MOLES	2.89135E-09	-0.
	MFRAC	5.19652E-11	-0.
H2O	MOLES	5.53436E 01	-0.
	MFRAC	9.94673E-01	-0.
NA+	MOLES	1.50000E-01	-0.
	MFRAC	2.69590E-03	-0.
LACTIC	MOLES	1.04000E-01	-0.
	MFRAC	1.86916E-03	-0.
CL-	MOLES	4.13951E-02	-0.
	MFRAC	7.43981E-04	-0.
PROTN	MOLES	9.99788E-04	-0.
	MFRAC	1.79725E-05	-0.
ACOOH	MOLES	-0.	2.58132E-05
	MFRAC	-0.	1.13057E-04
ACOO-	MOLES	-0.	1.03419E-03
	MFRAC	-0.	4.52955E-03
BCOOH	MOLES	-0.	4.66785E-03
	MFRAC	-0.	2.04443E-02
BCOO-	MOLES	-0.	1.00432E-01
	MFRAC	-0.	4.39875E-01
IMID+	MOLES	-0.	1.65672E-02
	MFRAC	-0.	7.25613E-02
IMID	MOLES	-0.	4.32804E-04
	MFRAC	-0.	1.89560E-03
AMIN+	MOLES	-0.	1.05610E-03
	MFRAC	-0.	4.62554E-03
AMIN	MOLES	-0.	3.89716E-06
	MFRAC	-0.	1.70688E-05
EAMIN+	MOLES	-0.	6.04980E-02
	MFRAC	-0.	2.64970E-01
EAMIN	MOLES	-0.	1.98966E-06
	MFRAC	-0.	8.71433E-06
PHENO	MOLES	-0.	2.01998E-02
	MFRAC	-0.	8.84714E-02
PHENO-	MOLES	-0.	2.03276E-07
	MFRAC	-0.	8.90314E-07
GUAN+	MOLES	-0.	2.34000E-02
	MFRAC	-0.	1.02488E-01
GUAN	MOLES	-0.	1.53548E-09
	MFRAC	-0.	6.72512E-09
NH3+CL	MOLES	-0.	-0.4.60488-03
	MFRAC	-0.	-0.2.016 -02
NH3+	MOLES	-0.	-0.1.01521-01
	MFRAC	-0.	-0.1.000 -00

essentially saturated with Cl^- at pH 5, so that, on the acid side, where k increases with the charge on the protein, these sites merely supply a bias of +4 to $\bar{\nu}_{\text{Cl}^-}$, the number of Cl^- bound. Further, for the 84 groups with $k = 1$, so few are bound (less than 2) at pH 5 and k increases so slowly with charge (Eq. (9)) that they affect the Cl^- binding curve only near pH 2. In particular, only 6 are bound at pH 3.0 using the Linderstrom-Lang theory and H^+ binding. The binding curves for these two subgroups are shown in Figs. 4c and d.

It follows from the above and from Fig. 4a, where approximately 7 total Cl^- are bound at pH 5 and 20 bound at pH 3, that a third subgroup of 10 to 12 $-\text{NH}_3^+$ sites are required wherein, on the average, 2 sites are binding Cl^- at pH 5 and practically all are saturated at pH 3. The sum of all three subgroups will then be a reasonably close fit to the empirical Cl^- binding data.

However, as Saroff points out [11], an additional hypothesis of molecular structure or function is required. By the Linderstrom-Lang theory alone, the binding constant k for any such subgroup of 12 sites does not increase fast enough with decreasing pH. A fit, however, can be obtained (Fig. 4f) by multiplying the Debye Huckel parameter, ω ,

for the 12 sites only, by a factor inversely proportional to pH--the 12 binding sites thus becoming much more effective as the pH decreases. Figure 4e shows such a saturation curve for these 12 sites, and Fig. 4f for the sum of all three subgroups. To rule out the possibility that the 12 sites are more effective over the entire range, ω for these sites was multiplied by a constant factor, 4.0, over the pH range (the result is plotted in Fig. 4g).

Various hypotheses can account for the variable affinity of these groups for Cl^- . For example, Saroff [11] proposes competitive H^+ binding which is now under investigation on the computer. Also, Tanford [26] proposes a reversible expansion of the protein molecule below pH 4, which could simply make more binding sites available. With only the Cl^- binding curve as evidence, it was not deemed defensible to speculate either way.

D. CALCIUM BINDING

On the alkaline side, positive ions may be expected to bind to serum albumin; in particular, as the protein becomes less positive Ca^{++} binds along an approximately exponentially increasing curve. Of the several studies of Ca^{++} binding, the data of Saroff and Lewis [34] gives

a clear empirical binding curve and is confirmed for the most part by Loken et al. [35], who also show the variation of apparent pK with pH, and the variation of percent free Ca^{++} with pH and total Ca^{++} concentration. McLean and Hastings [36] is the classic study in the field, relating Ca^{++} binding to human physiology.

On the basis of data in which the only variable is dilution, McLean and Hastings rule out the expected dissociation binding equation, viz.,

$$\frac{[\text{Ca}^{++}][\text{Prot}^-]^2}{[\text{Ca Prot}]} = k \quad (13)$$

in favor of

$$\frac{[\text{Ca}^{++}][\text{Prot}^-]}{[\text{Ca Prot}]} = K , \quad (14)$$

which suggests that particular pairs of sites acting as a unit are responsible for binding the Ca^{++} ion. Saroff [33] presents evidence that the binding pair are probably not both COO^- groups or not both NH_2 groups, but probably a COO^- and an NH_2 propitiously arranged geometrically and acting in concert, as in chelation or salt-bridging.

The difficulty in theoretical structure again lies in the fact that the binding curve is not purely exponential; in particular, the points near pH 7 to pH 8 appear to be significantly out of line on a log-log plot. However, on the alkaline side, not only is the charge on the molecule changing, changing K , but also more NH_2 groups are becoming available for binding Ca^{++} , as they apparently do [34]. In addition, at the isoelectric point approximately 5 percent of the carboxyl groups are still in the COOH form so that these sites, if they are effective, also become available. Clearly, therefore, Eq. (14) need not yield an exponential binding curve.

In Eq. (14) the meaning of the symbol $[\text{Pr}^{\equiv}]$ is in question so that the definition of K is not clear. Here we use the following: consider, as an example, only the binding to the carboxyl groups, of which there are approximately 105 at molecular weight 69,000. The groups are paired arbitrarily, and then considered to have the same concentration as though they were free in solution; i.e., with protein concentration at 1.0 mM, the carboxyl pairs have concentration $[\text{Pr}^{\equiv}] = 52.5$ mM.

As a first approximation to Ca^{++} binding, the McLean and Hastings model, in which pairs of carboxyl groups bind

a Ca^{++} ion, was used. The equation for Ca^{++} binding was added to the mathematical model along with Cl^- and H^+ binding. Ca^{++} was added as a component, 10 mm being added and a corresponding 20 mm of Na^+ deducted (approximately the amount used by Saroff [11]). The overall level of binding was adjusted by varying K at a calibration point, here pH 5.35. At pH 7.35 and 25°C , K should have a value 2.18 by Ref. 34 and 2.22 by Ref. 35. Then, with the Linderstrom-Lang equation corrected for the valence of calcium, the system was titrated from pH 5.35 to pH 10 to determine the shape of the binding curve. The apparent value of K varies with charge over this range, as well as with the binding sites available.

Figure 5a is a reproduction of the Saroff and Lewis [34] binding data with an approximate curve drawn through the points. Figure 5b is the computed binding curve obtained as described above (i.e., binding only to the paired carboxyl sites); it is a surprisingly good fit to the empirical data in the lower range of pH. The binding increases along an approximately exponential curve to pH 7.5, after which the rate of increased binding with pH is lower, as though the binding characteristics had changed. In fact, all of the carboxyl sites are finally in the COO^- form.

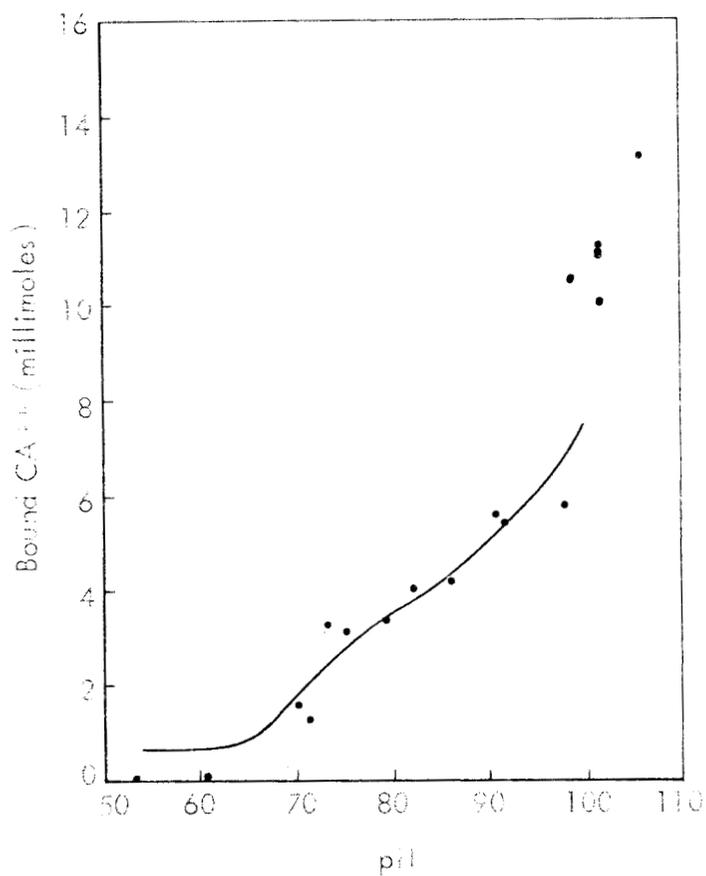


Fig. 5--Calcium binding to serum albumin:
a) Saroff and Lewis [35] (· points),
b) Computed curve binding to carboxyl groups only (solid line)

In view of this rather good fit to the empirical data up to pH 9, the salt-bridging experiments were deferred. Evidently, however, in the vicinity of pH 10 some effects other than Eqs. (9) and (14) are responsible for the increased binding, and in this region the probability of occurrence of $\text{NH}_2\text{-COO}^-$ pairs is increasing. By supposing two such pairs, the binding of Ca^{++} at pH 10 could be increased to match the empirical data. However, because the binding to COO^- sites alone fits the non-analytic curve to pH 8.5, it may be assumed that such paired sites begin to appear in the alkaline range above pH 8.

The binding of Ca^{++} to paired carboxyl sites alone gives rise to an equation of the form (14) for which K is a function of pH. With the definition of $[\text{Pr}^-]$ as above, and with $[\text{CaPr}]$ equal to the concentration of the conceptual group $\text{Ca}(\text{COO}^-)_2$ at pH 7.35, we have

$$\frac{[\text{Ca}^{++}][\text{Pr}^-]}{[\text{Ca Pr}]} = \frac{0.008572 \times 0.05155}{0.001428} = 0.3094 \quad (15)$$

so that at pH 7.35, $pK = 0.51$. Since, in this calculation, one liter of solvent is used (25°C), the concentrations are approximately molal. If the solvent is reduced to give one liter of solution, the molar concentration of total

Ca is 10.0, and the pK at 7.35 shifts to 0.505, not much change. On the other hand, if [CaPr] is taken to be the concentration of the protein molecule, the pK at 7.35 is 2.217, while Ref. 36 gives 2.2. Also, the pK increases with pH (as Ref. 35 shows).

V. DISCUSSION

With any of the above procedures, binding Cl^- or Ca^{++} , the empirical H^+ titration curve is, of course, exactly fitted by the calculation, since, with the Linderstrom-Lang modification of the pK , all of the variation occurs in the Debye-Huckel parameter ω . Figure 2c is a plot of ω versus pH with Cl^- and Ca^{++} binding. Again, the calculation for ω near the isoionic point is imprecise, but this inaccuracy is not directly pertinent since it is supposed that the molecule is stable in this range, and greater precision is possible if required.

The more interesting aspect is the stability of ω in the extreme ranges. The calculated ω bears little resemblance to that found experimentally (i.e., without the computer); but, in fact, under the conditions described for the computer experiment below pH 4.5 and above pH 7.0, the calculated ω is highly accurate. Further, it is difficult to see how the developing experiments in salt-bridging and chelation can change ω by more than a few percent. More likely, because of the insensitivity of ω , it is the wrong choice of parameter on which to base speculation on molecular configuration. More sensitive

tests of molecule geometry may be based on attempts to fit empirical data directly, e.g., considering hypothetical individual site reactions as in the work of Saroff [11,34].

The motivation for this experimental model was to obtain an "effective" titration curve (Fig. 1d) for use in a larger system, but the methods are quite general and may prove useful elsewhere. It is possible, for example, to apply the techniques of calculation to other chemical systems and thus expose for study the details of complex reactions.

Various experiments have suggested themselves during this study: the variation of the "intrinsic" pK with ionic strength; a more precise analysis of the system near the isoelectric point; the implications of a non-spherical molecule; a molecule penetratable by water molecules; and the general subject of salt-bridging. Further work from a purely theoretical view, however, might more appropriately be directed toward the question of whether the Linderstrom-Lang theory is still adequate. As Tanford and Kirkwood [15] suggest, it is probably now possible to compute the titration curve for a protein directly from the geometrical coordinates of the sites within the molecule. This larger problem is now under consideration.

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