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Binding of Sodium Ions to β-Lactoglobulin*

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ABSTRACT: Measurements on the binding of Na^+ by the allelic forms A and B of β-lactoglobulin reveal no difference in the binding properties of the two forms. No Na^+ is bound at the isoionic point, despite a decrease in *p*H upon addition of NaCl to the deionized crystalline protein at this point. With increasing *p*H, the binding of Na^+ increases to the value of 4.28 per mole of β-lactoglobulin at *p*H 9.48 when the total concentration of sodium ion is 0.06 M. Above *p*H 9.5 the protein becomes irreversibly denatured. A model involving chelation of Na^+ between four carboxyl groups and the four imidazole side chains of the molecule is evoked, and the equation predicting the course of the binding deduced from this model is derived. A representative best fit yields an association constant, k_{Na}, of 150 for the binding of Na^+ to β-lactoglobulin. The calculated curve is compared with previously published optical rotation data in this *p*H region with good fit, from which the conclusion is drawn that both the binding of Na^+ and the configurational change, presumably unfolding, appear to be controlled by the same ionization reaction.

β-Lactoglobulin is one of the few proteins which bind sodium ions. The extent of the binding of Na^+ has been measured previously at *p*H 7.4 (Carr, 1956) and extrapolation of these data to the isoionic *p*H of 5.3 has been used as evidence of the binding of Na^+ or K^+ at the isoionic point as a mechanism for the lowering of *p*H of deionized solutions of β-lactoglobulin upon addition of NaCl or KCl (Nozaki *et al.*, 1959; Tanford and Nozaki, 1959). Studies of other proteins which bind Na^+ or K^+, particularly myosin, have been interpreted by a mechanism involving the chelation of the cation by carboxyl and imidazole or amino groups, thus implying a degree of three-dimensional structural specificity in the protein (Lewis and Saroff, 1957; Saroff, 1957a). In order to investigate this subject in greater detail, the study of the *p*H dependence of Na^+ binding to β-lactoglobulin was undertaken.

Experimental Procedure

Source, Isolation, and Crystallization of Protein. Milk from cows known to be homozygous for either the A or B form of β-lactoglobulin was obtained through the generosity of Dr. C. A. Kiddy of the Agricultural Research Station, Beltsville, Md. The isolation procedure of Aschaffenburg and Drewry (1957) was used, and crystallization was accomplished by deionizing a 0.25% protein solution with a Dintzis (1952) column. Crystals of β-lactoglobulin A formed spontaneously, but crystallization of β-lactoglobulin B was accomplished only after seeding deionized solutions with crystals of this form kindly furnished by Dr. R. Townend of the Eastern Regional Research Laboratory, Philadelphia, Pa. No difference in Na^+ binding was observed between β-lactoglobulin A crystallized twice, as opposed to once, and ultracentrifugation analysis revealed a single symmetrical peak. β-Lactoglobulin B was crystallized only once in view of these results.

Preparation of Protein Solutions. All protein solutions were made by dissolving deionized crystalline protein with dilute NaOH to reach the desired *p*H, and then adjusting the free Na^+ concentration to approximately 0.05 M with NaCl. NaOH solutions were standardized against standard HCl and H_2SO_4 solutions, in turn referred to potassium acid phthalate as a primary standard. NaOH standards were kept in an atmosphere of N_2 and restandardized just prior to each series of measurements. Standard NaCl solutions were made from ACS analytic reagent grade NaCl, dried at 105° for 24 hours. Deionized water was used throughout. Protein concentration measurements were made at 278.5 mμ on a Beckman DU spectrophotometer, using an optical density $E^{1\%}_{1cm}$ value of 9.66, which was determined by dry-weight analysis of three samples of deionized β-lactoglobulin A after exposure to 105° for 24 hours. A

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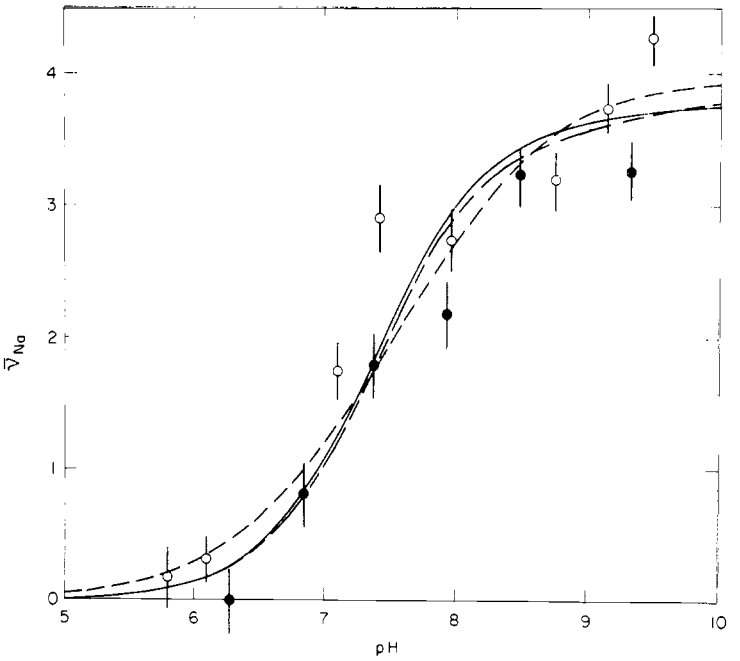


FIGURE 1: Experimental γ_{Na} versus pH. O, A form; ●, B form; ———, calculated representative best fit to the hand curve, with $k_{H_2} = 10^{7.0}$, $k_{Na} = 150$, $\epsilon_H(1 + k_D) = 19$, $w = 0.02$, $\sigma = 0.0437$; - - - -, linear logarithmic plot fitted by the method of least squares; - - - - -, the machine best fit to (- - - -), using equation (7) with $n = 4$, $k_{H_2} = 10^{7.5}$, $k_{Na} = 50$, $\epsilon_H(1 + k_D) = 21$, $w = 0.05$, $\sigma = 0.134024$.

molecular weight of 35,500 and a partial specific volume of 0.7514 cc/g were assumed for both forms of the protein.

Measurement of Free Na^+ *and* Cl^- *Concentrations.* Binding measurements were made by the methods of Carr (1953) and Scatchard *et al.* (1959) with an apparatus previously described (Saroff and Healy, 1959) consisting of three chambers in a linear array, separated in pairs by two permselective membranes, one negatively charged (sulfonic acid resin) and the other positively charged (quaternary amine resin). The membranes, identified as Nalfilm 1 and Nalfilm 2, were supplied by the National Aluminate Corp., Chicago, Ill. Placing the protein solution in the center chamber and accurately known standard solution of NaCl in the end chambers created a potential across each membrane, linearly related to the logarithm of the activity of the free Na^+ or Cl^- in the protein solution. Standard solutions were chosen such that the absolute magnitude of the measured potentials seldom exceeded 3 mv. Equilibration of the solutions required from 5 to 10 minutes. These potentials were recorded by formation of a liquid junction between the solutions and a saturated KCl solution, thence to calomel electrodes and a Cary Model 31 vibrating-reed electrometer. A linear interpolation to zero potental on a graph of millivolts versus log Na^+ or Cl^- gives the log free Na^+ or Cl^-. The difference between Na^+ added and free Na^+ measured is the Na^+ bound to the protein. Activity coefficients were assumed to be the same on both sides of the membrane. All measurements were made at 25 ± 1°. The pH measurements were made on a Beckman Model G pH meter standardized to the nearest integral pH value.

Experimental Results

Table I and Figure 1 exhibit the experimental data. The symbol $\bar{\nu}_{Na}$ represents the moles of Na^+ bound per mole of protein, σ equals the experimental standard deviation, and Z_P is the net charge on the protein resulting from dissociation of H^+ and binding of Na^+, measured from the isoionic point at pH 5.3 where $Z_P = 0$. The A form of the protein contains two carboxyl groups more per molecule than form B (Gordon *et al.*, 1961; Piez *et al.*, 1961), but for purposes of calculation the isoionic point of both forms was taken to be the same, and the value of Z_P at all pH values was taken to be that of the normal protein, i.e., that of a 1:1 mixture of the A and B forms. This is justified since the maximum difference in the calculated curves between the A and B forms occurs at pH 10, where Z_P reaches a maximum value, at which point $\Delta\bar{\nu}_{Na} = 0.013$. The solid curve is the calculated representative best fit, the dashed one that given by the method of least squares. There is no discernible difference in $\bar{\nu}_{Na}$ between the A and B forms of the protein within the limits of precision of the methods used. A pH change upon addition of NaCl to deionized crystals of the protein was observed, in general agreement with that previously published by Tanford and Nozaki (1959).

TABLE I: Summary of Data for the Binding of Na^+ to β-Lactoglobulin.

Protein Concn (M × 10^3)	Protein Form	pH	OH^- Added per Mole of Isoionic β-Lacto-globulin	Total Concn Na^+ (M × 10^2)	Total Concn Cl^- (M × 10^2)	Free Ion Concn Na^+ (M × 10^2)	Free Ion Concn Cl^- (M × 10^2)	Sodium Ions Bound $\bar{\nu}_{Na} \pm \sigma$	Net Charge of β-Lacto-globulin Z_p
1.44	A	5.79	5.93	5.101	4.247	5.08	4.28	0.17 ± 0.24	−5.76
1.90	A	6.09	7.96	5.011	3.498	4.95	3.43	0.31 ± 0.18	−7.65
1.34	B	6.27	7.37	4.949	3.962	4.95	4.01	0.00 ± 0.26	−7.37
1.36	B	6.84	10.11	5.139	3.764	5.03	3.76	0.82 ± 0.25	−9.29
1.90	A	7.10	12.94	6.517	4.059	6.18	4.07	1.75 ± 0.23	−11.2
1.33	B	7.37	13.01	5.212	3.482	4.97	3.46	1.80 ± 0.26	−11.2
1.30	A	7.42	14.04	5.589	3.632	5.21	3.67	2.91 ± 0.27	−11.1
1.37	B	7.93	13.90	5.438	3.535	5.14	3.53	2.19 ± 0.25	−11.7
1.54	A	7.96	16.03	5.540	3.072	5.12	3.10	2.74 ± 0.22	−13.3
1.33	B	8.48	16.84	5.423	3.183	4.99	3.15	3.25 ± 0.26	−13.6
1.54	A	8.76	20.03	5.617	2.532	5.12	2.56	3.20 ± 0.23	−16.8
1.90	A	9.15	19.92	10.00	6.219	9.29	6.24	3.75 ± 0.19	−16.2
1.35	B	9.33	19.90	5.561	2.854	5.12	2.88	3.27 ± 0.26	−16.6
1.90	A	9.48	23.88	6.000	1.462	5.19	1.51	4.28 ± 0.19	−19.6

Error Analysis.[1] This analysis is based on the assumption that the variance of the measurements of the Cl^- concentrations is equal to that of the Na^+ measurements. This seems a reasonable assumption, since the measurements were made simultaneously on the same solution with the same instruments by the same observer.

Assuming the random errors occurred following a Poisson distribution (Schneiderman and Brecher, 1950) and that there is zero correlation between the Na^+ and protein concentration measurements, we have the equation

$$V(X/Y) = (X^2/Y^2)[V(Y)/Y^2 + V(X)/X^2] = 2$$

Here X = (total concentration of Na^+) − (free concentration of Na^+), Y = protein concentation, $V(\ )$ is the variance of the quantity in parentheses, and σ is the experimental standard deviation. Then $V(X)$ = V(total concentration of Na^+) − V(free concentration of Na^+) = V(total concentration of Cl^-) − V(free concentration of Cl^-), where again zero correlation has been assumed between total Na^+ and free Na^+ determinations. An error of 1.5% is taken for the protein concentration measurement, i.e., $V(Y)$ = $(0.015Y)$. Attempts were made to fit the data by: (1) a polynomial of high degree, (2) casting equation (6) into logarithmic form which yielded a linear equation, and (3) plotting the experimental points as a Gaussian and as a logistic distribution, both of which gave linear plots. The polynomial curve was an obvious misfit. For the linear plots, the method of least squares was used to fit a first degree polynomial, weighing each point with the variance involved in the determination of that point. The sigmoid curves corresponding to these straight lines on the $\bar{\nu}_{Na}$ versus pH plot were flatter than and shifted to the right by 0.1–0.2 pK unit when compared to the hand-drawn curve; i.e., $k_{H_2} = 10^{7.1}$–$10^{7.2}$. The process of transforming the data into the linear forms also required ignoring completely the points at 6.27 and 9.48. For these reasons (together with the fact that a least-squares fit to the linear form is not necessarily the best fit to the curve) plus the fact that the hand-drawn curve appeared to describe the data best, we chose the latter as our standard curve.

The only factors not taken into account by this analysis are: (1) differences in behavior of the two membranes, shown to be vanishingly small by calibration prior to each series of measurements; and (2) errors in the volumetric and gravimetric techniques which were at least one order of magnitude less than the random errors.

Interpretation of Results

Carboxyl Groups. Most proteins containing carboxylate ions as their charged acidic groups do not bind sodium ions. The few proteins containing only carboxylate ions as their acidic groups which do bind sodium ions are β-lactoglobulin, zein (Carr, 1956), and myosin (Lewis and Saroff, 1957). The binding of sodium ions to these proteins can be cast into the electrostatic framework of the Linderstrøm-Lang (1924) model where the number of sodium ions bound per mole of protein, $\bar{\nu}_{Na}$, is given by the expression

[1] We wish to thank Dr. Marvin Schneiderman for guidance in this analysis.

$$\bar{\nu}_{Na} = \frac{k_{Na}' c_{Na} n}{1 + k_{Na}' c_{Na}} \quad (1)$$

where

$$k_{Na}' = k_{Na} \exp(-2Z_P w Z_H) \quad (2)$$

and k_{Na} refers to the intrinsic association constant for the binding of sodium ions to n independent carboxylate ions, c_{Na} is the concentration of free sodium ions in solution, Z_P is the net charge on the protein, Z_{Na} is the charge on the sodium ion, and w is the electrostatic interaction factor. The binding data can be explained with this model for β-lactoglobulin with the values $k_{Na} = 0.45$, $w = 0.08$, and $n = 50$ (50 carboxyl groups per mole of β-lactoglobulin). With this model the value of $\bar{\nu}_{Na}$ is a bit too high in the region of *p*H 5.0, the value of $\bar{\nu}_{Na}$ rises too rapidly between *p*H 9.0 and 9.5, and the value of k_{Na} is too large. Most important, however, is the fact that within the framework of this model (with $k_{Na} = 0.4$) most proteins should bind sodium ions. Since only a small number of proteins bind sodium ions, the chelation mechanism previously proposed for myosin (Lewis and Saroff, 1957; Saroff, 1957a) is employed for β-lactoglobulin.

For the chelation model consider the following reactions involving pairs of ionizable carboxyl and nitrogen groups:

$$\begin{aligned}
&\text{(A)}\quad \text{∫—COO}^-, \text{N—∫} + \text{H}^+ = \text{∫—COOH, N—∫} && k_{H_1} = \frac{P(COOH, N)}{P(COO^-, N)\, c_H}\\
&\text{(B)}\quad \text{∫—COO}^-, \text{H}^+\text{N—∫} + \text{H}^+ = \text{∫—COOH, H}^+\text{N—∫} && k_{H_1} = \frac{P(COOH, H^+N)\epsilon_H}{P(COO^-, H^+N) c_H}\\
&\text{(C)}\quad \text{∫—COOH, N—∫} + \text{H}^+ = \text{∫—COOH, H}^+\text{N—∫} && k_{H_2} = \frac{P(COOH, H^+N)}{P(COOH, N) c_H}\\
&\text{(D)}\quad \text{∫—COO}^-, \text{N—∫} + \text{H}^+ = \text{∫—COO}^-, \text{H}^+\text{N—∫} && k_{H_2} = \frac{P(COO^-, H^+N)}{P(COO^-, N) c_H \epsilon_H}\\
&\text{(E)}\quad \text{∫—COO}^-, \text{H}^+\text{N—∫} = \text{∫—COO}^- \ldots \text{H}^+ \ldots \text{N—∫} && k_D = \frac{P(COO^- \ldots H^+ \ldots N)}{P(COO^-, H^+N)}\\
&\text{(F)}\quad \text{∫—COO}^-, \text{N—∫} + \text{Na}^+ = \text{∫—COO}^- \ldots \text{Na}^+ \ldots \text{N—∫} && k_{Na} = \frac{P(COO^- \ldots Na^+ \ldots N)}{P(COO^-, N) c_{Na}}
\end{aligned} \quad (3)$$

where ∫—COOH represents a terminal or side-chain carboxyl group, and H⁺N-I stands for an imidazole, α- or ε-amino, or guanidino group. The corresponding dissociated forms are ∫—COO⁻ and N—∫. The symbol ∫—COO⁻ . . . H⁺ . . . N—∫ represents a hydrogen-bonded species, and ∫—COO⁻ . . . Na⁺ . . . N—∫ indicates a species involving a chelated Na⁺. Here k_{H_1} is the unperturbed equilibrium constant for the association reaction of equation (A), and ϵ_H in equation (B) takes into account the local electrostatic repulsive force between the H⁺ and H⁺N structures. Similarly, k_{H_2} refers to the unperturbed association constant of equation (C), and $1/\epsilon_H$ then measures the local electrostatic effect of a neighboring COO⁻ group interacting with the approaching H⁺ in equation (D). The constant for hydrogen bond formation is k_D, that for formation of the Na⁺ chelate is k_{Na}, and c is the concentration of the subscripted ion. P(COO⁻, N) represents the fraction of a single pair of one protein molecule existing in the form ∫—COO⁻, N—∫, and similar definitions hold for the remaining P( ) symbols.

Obviously

$$\Sigma P(\ ) = 1 \quad (4)$$

Expression of each P( ) fraction in terms of P(COO⁻ . . . Na⁺ . . . N) and the equilibrium constants from equations (3) enable one to substitute them in equation (4), whence after multiplication by n, the maximum value of $\bar{\nu}_{Na}$, we have

$$\bar{\nu}_{Na} = nP(COO^- \ldots Na^- \ldots N)$$

$$\bar{\nu}_{Na} = \frac{nk_{Na}c_{Na}}{1 + k_{H_1}c_H + k_{H_2}c_H[\epsilon_H(1 + k_D) + k_{H_1}c_H] + k_{Na}c_{Na}} \quad (5)$$

Introduction of generalized electrostatic effects dependent upon the net charge of the protein molecule, Z_P, the charges of the interacting ion, Z_H and Z_{Na}, and the Linderstrøm-Lang (1924) electrostatic interaction factor w, is accomplished by equations (2) and (6):

$$k_H' = k_H \exp(-2Z_P w Z_H) \quad (6)$$

where the unprimed k's refer to the intrinsic equilibrium constants. Since $Z_H = Z_{Na} = 1$, equation (5) becomes

$$\bar{\nu}_{Na} = \frac{nk_{Na}c_{Na}}{\exp(2Z_P w) + k_{H_1}c_H + k_{H_2}c_H[\epsilon_H(1 + k_D) - k_{H_1}c_H \exp(-2Z_P w)] + k_{Na}c_{Na}} \quad (7)$$

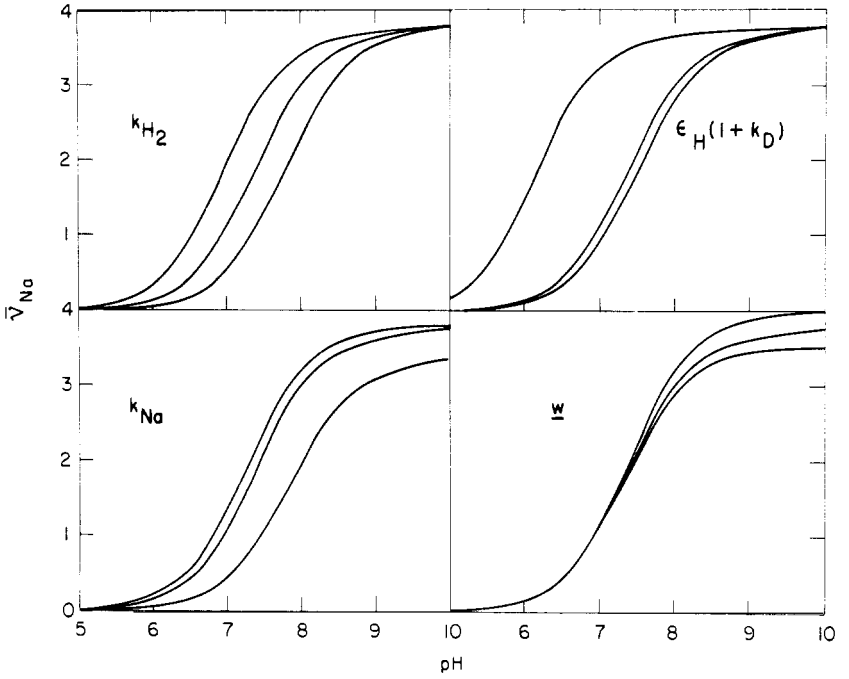


FIGURE 2: Calculated curves showing the effect of individual variables on equation (7). In each instance, from left to right: upper left figure, $k_{H_2} = 10^{6.6}$, $10^{7.0}$, $10^{7.4}$; upper right figure, $\epsilon_H(1 + k_D) = 1$, 19, 25; lower left figure, $k_{Na} = 200$, 150, 50; lower right figure, $w = 0.10$, 0.02, 0. The center curve in each instance is the same representative best fit to the hand curve, with values as in Figure 1.

Curve Fitting. In order to fit this equation to the experimental data, a smooth curve was fitted by hand to the middle of the range defined by the experimental points for use as a standard curve for approximation by equation (7) with $n = 4$ (see comment under Error Analysis). The range of values of the variables was chosen as follows: $k_{H_1} = 10^{4.8}$ (Tanford, 1962); k_{Na} = 50, 100, 150, and 200 because of data of Table II (Saroff, 1957a) as well as preliminary experiments indicating a value of *ca.* 100; $w = 0$, 0.01, 0.02, 0.05, 0.07, and 0.1 to cover the entire range of w encountered previously in this field; and $\epsilon_H(1 + k_D) = 1$, 3, 5, ..., 23, 25 to cover reasonable values for the hydrogen-bonding association constant (Scheraga, 1961). The constant k_{H_2} was assigned the values $10^{6.6}$, $10^{6.8}$, $10^{7.0}$ $10^{7.2}$, and $10^{7.4}$, since it was apparent from Figure 1 that these values would bracket the standard curve. Calculation of curves from equation (7) using all combinations of the foregoing values of the variables was accomplished by means of a Minneapolis-Honeywell 800 computer.[2] The closeness of fit to the standard curve was determined by the expression:

$$S_Y = \text{standard error of estimate} = (\Sigma d_i^2/N)^{1/2}$$

where

$$\Sigma d_i^2 = \Sigma(\bar{\nu}_{istd} - \bar{\nu}_{icalcd})^2,\ i = 1,\ldots,11$$

for $N = 11$ points. For each value of S_Y, a number of curves approximated the standard one, since the latter is not sufficient to uniquely determine the variable involved. Choosing the value $S_Y = 0.052$ eliminated all but the eight combinations listed in Table III, and from these values $k_{H_2} = 10^{7.0}$, $w = 0.02$, $k_{Na} = 150$, and

TABLE II: Association Constants for Na^+ Chelates.

Chelating compound	Log k
Ethylenediaminetetraacetic acid	1.66[a]
Aminobarbituric acid–*N*,*N*-diacetic acid	3.32[b]
Ammoniatriacetic acid	2.15[c]
Carboxylimidazole chelate (myosin)	3.2[d]
Carboxylamino chelate (myosin)	2.6[d]

[a] Schwarzenbach and Ackermann (1947). [b] Schwarzenbach *et al.* (1946). [c] Schwarzenbach *et al.* (1945). [d] Lewis and Saroff (1957).

[2] We are indebted to Mr. Marvin Shapiro for instruction in FORTRAN programming and for guidance in its application to the Honeywell 800 computer.

TABLE III: Values of Parameters for Equation (7) for $n = 4$.

k_{H_2}	k_{Na}	w	$\epsilon_H(1 + k_D)$	S_Y ($\times 10^2$)
$10^{6.6}$	50	0.05	15	3.92
$10^{6.8}$	50	0.05	9	3.56
$10^{7.0}$	150	0.02	17	5.04
$10^{7.0}$	150	0.02	19	4.37
$10^{7.0}$	200	0.01	25	4.27
$10^{7.2}$	150	0.02	11	4.47
$10^{7.2}$	200	0.01	15	4.37
$10^{7.4}$	150	0.02	7	4.27

$\epsilon_H(1 + k_D) = 19$ were chosen as reasonable values to give a representative best fit.

Effect of Individual Variables. The effects of the variables of equation (6) are best shown by Figure 2, where in each case the center curve is the representative best fit, and the outer curves are given by the extreme values of the ranges listed. Increase of k_{H_2} shifts the entire curve to the right by an amount equal to the increase, when measured along the line $\bar{\nu}_{Na} = 2$. Increase of $\epsilon_H(1 + k_D)$ has a similar effect. Increasing k_{Na} has a moderate effect, pushing the main portion of the curve to the left and elevating the terminal portion at high *p*H. Here w has no effect below *p*H 7.5 and only a slight elevating effect again on the terminal portion at high *p*H; k_{H_1} has little effect, the maximum change being $\Delta\bar{\nu}_{Na} = 0.014$ between the combination $k_{H_1} = 10^{5.0}$, $k_{H_2} = 10^{7.0}$, and $k_{H_1} = 0$, $k_{H_2} = 10^{7.0}$. It was included, since it refers to a group essential to our model for chelation.

Nitrogen Group Identification. IMIDAZOLE. The identification of the nitrogen atom of the chelation phenomenon as a member of an imidazole residue is apparently reasonable. The value of k_{H_2} is that expected from previous data. In addition, the maximum number of Na^+ bound per molecule of protein is four, and there exist four imidazole groups in such a molecule. Similar carboxyl-imidazole or carboxyl-amino chelates have been postulated for myosin.

AMINO. That the nitrogen atom may be in an α- or ϵ-amino group is possible, but the k_{H_2} value is not consistent with those previously recorded for these groups. Using values of k_{H_2} of $10^{8.2}$, $10^{8.4}$, and $10^{8.6}$, the lower end of the range expected for the intrinsic constants of such groups, further calculations varying $\epsilon_H(1 + k_D)$ reveal that only one combination gives a reasonable fit. This combination is: $k_{H_1} = 10^{4.8}$, $k_{H_2} = 10^{8.2}$, $k_{Na} = 150$, $w = 0.02$, and $\epsilon_H(1 + k_D) = 1$. As k_{H_2} increases above this value, S_Y increases rapidly, thus ruling out higher values for this most important variable. The value of $\epsilon_H(1 + k_D) = 1$ implies that both the local electrostatic effect and hydrogen bond are nonexistent.

GUANIDINO AND PHENOLIC GROUPS. The nitrogen atom obviously cannot be in a guanidino group, since this group only ionizes in the *p*H range above 12, and the nitrogen is unlikely to be substituted for by the phenolic oxygen atom of a tyrosine residue since the *pK* for its ionization is in the neighborhood of 10.

Thus the reasonable chelates are those involving the carboxyl-imidazole and carboxyl-amino pairs. The values required for each of these pairs are summarized in Table IV. Within the limits of holding k_{H_2} below $10^{8.2}$, the value of $\epsilon_H(1 + k_D)$ may be shifted to hold equation (6) to a good fit with the experimental data. We prefer the carboxyl-imidazole pair mainly because the value of $\epsilon_H(1 + k_D) = 19$ is consistent with the values of ϵ_H and k_D arrived at for serum albumin (Saroff, 1957b; Saroff and Lewis, 1963).

TABLE IV: Comparison of Values of Equation (7) for Carboxylimidazole and Carboxylamino Pairs.

	Carboxyl-imidazole	Carboxyl-amino
k_{H_1}	$10^{4.8}$	$10^{4.8}$
k_{H_2}	$10^{7.0}$	$10^{8.2}$
k_{Na}	150	150
w	0.02	0.02
$\epsilon_H(1 + k_D)$	19	1

Discussion

Correlation with Optical Rotation Data. Tanford *et al.* (1959) and Tanford and Taggart (1961) have published optical rotation data for β-lactoglobulin as a function of *p*H. These data are plotted in Figure 3, upon which is superimposed the representative best-fit curve calculated previously. There is a close fit of the curve to the data, and some relationship may exist between the Na^+ binding and the configurational change believed to be occurring. Tanford *et al.* (1959) proposed that this change was an unfolding phenomenon.

The fall in *p*H on the addition of salt to isoionic β-lactoglobulin and the data reported herein indicating no binding of Na^+ to β-lactoglobulin in the region of *p*H 5 are inconsistent within the framework of the electrostatic model of Linderstrøm-Lang (1924) as applied by Nozaki *et al.* (1959). With no binding of sodium ions to β-lactoglobulin in the region of *p*H 5, some mechanism other than that of an electrostatic effect must be hypothesized for the fall in *p*H on the addition of salt. The observed fall in *p*H could also result from a dissociation reaction involving aggregates of β-lactoglobulin wherein some of the carboxyl groups were affected either by taking part directly in, or by having their *pK* values shifted by, an aggregation reaction.

Comparison of the Binding of Sodium and Calcium

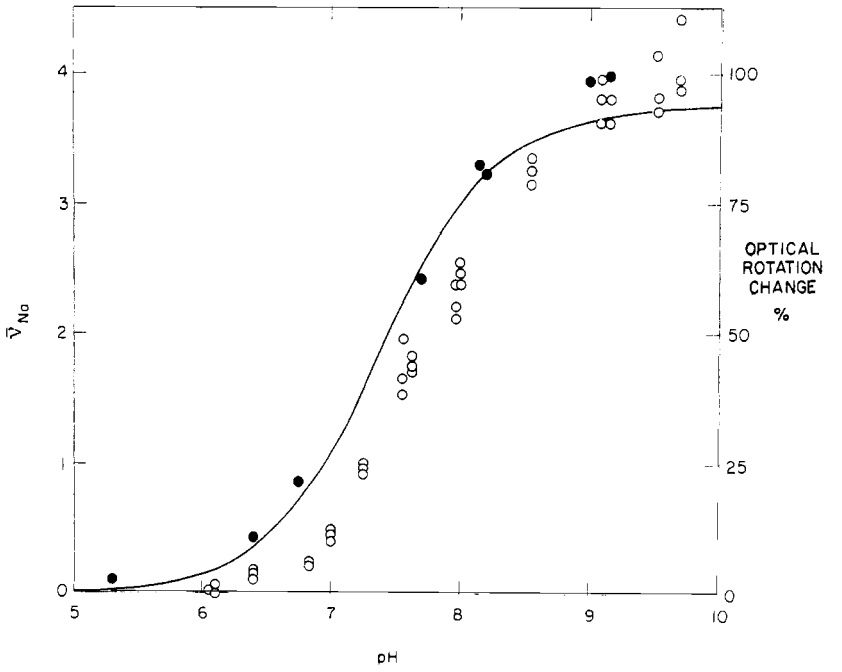


FIGURE 3: Optical rotation change data. O, data from Tanford and Taggart (1961); ●, data from Tanford *et al.* (1959). Curve is the calculated representative best fit to the hand curve (with values as in Figure 1) to the Na^+ binding data of Figure 1.

TABLE V: Constants for the Binding of Ca^{2+} and Na^+ to various Ligands.

Ligand	Ca^{2+}	Na^+
Ammonia	0.6	
Imidazole	2.0	
Monocarboxyllic acids	1.0–6.3	
Succinic acid	10–100	
Glycolic acid	13–39	
Glycine	22–27	
Maleic acid	12–270	
Oxalic acid	1000	
Citric acid	2000	
Methylamine-*N*,*N*-diacetic acid	5600	
Ethylenediamine-*N*,*N*-diacetic acid	$10^{4.6}$	
Ammoniatriacetic acid	$10^{5.2}$	126
Aminobarbituric acid–*N*,*N*-diacetic acid	$10^{8.7}$	2000
Ethylenediaminetetraacetic acid	$10^{10.6}$	50

Ions to Proteins. Although the binding of sodium ions to proteins is relatively rare and the binding of calcium ions is relatively common, the *p*H dependence of the binding of both of these cations is approximately the same in the proteins serum albumin, myosin, and β-lactoglobulin. Myosin binds both sodium ions (Lewis and Saroff, 1957) and calcium ions (Nanninga, 1957); β-lactoglobulin also binds both sodium ions and calcium ions (Carr, 1956); serum albumin binds calcium ions (Saroff and Lewis, 1963) but does not bind sodium ions (Lewis and Saroff, 1957).

Sodium ions are considered to have a coordination number of four while calium ions usually exhibit a coordination number of six (Martell and Calvin, 1952). Unidentate ligands give association constants for Ca^{2+} varying from about 1 to 10. Bidentate ligands give association constants for Ca^{2+} varying from 10 to 1000. The possible tridentate ligand, citric acid, has a constant of 2000. Ligands capable of forming tetradentate and higher-order complexes give association constants above 10^4 (see Table V, and Bjerrum *et al.*, 1957). As illustrated in Table V, the ligands capable of forming the tetradentate and higher-order complexes with Ca^{2+}

TABLE VI: Constants for the Binding of Na^+ and Ca^{2+} to Proteins.

	Ca^{2+}	Na^+
Serum albumin	10–20	0
β-Lactoglobulin		150–200
Myosin	$10^{4.7}$–$10^{5.3}$[a]	400–1600

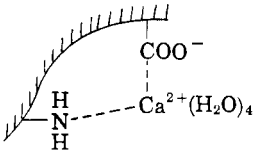
[a] Values of Nanninga (1957) corrected for *pK* of 9.7 for hydrogen binding.

also complex Na^+ with constants ranging from 50 to 2000.

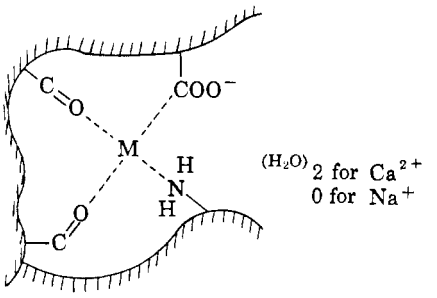
Constants for the binding of Ca^{2+} and Na^+ to serum albumin, β-lactoglobulin, and myosin are listed in Table VI. From the constants in Table VI we propose that serum albumin binds Ca^{2+} in a bidentate complex and that myosin binds Ca^{2+} in at least a tetradentate complex. A bidentate structure would thus be insufficient to bind Na^+ while the tetradentate structure would provide a constant of sufficient size to bind Na^+.

With the assumption that the same sites in these proteins are responsible for the binding of both Na^+ and Ca^{2+}, we propose the following structures for the binding of Ca^{2+} to serum albumin and Ca^{2+} and Na^+ to β-lactoglobulin and myosin:

serum albumin:



β-lactoglobulin and myosin:



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