A STUDY OF SELECTIVE ADSORPTION OF Na^+ AND OTHER ALKALI-METAL IONS ON ISOLATED PROTEINS: A TEST OF THE SALT-LINKAGE HYPOTHESIS

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. *According to the salt-linkage hypothesis (a part of the association-induction hypothesis), the binding of alkali-metal ions on isolated proteins may be low as a result of the masking of cation-binding fixed anionic groups by the formation of "salt linkages" between them and fixed cationic groups. This hypothesis has been verified in a quantitative manner by the measurement of free and adsorbed Na^+ in solutions of bovine hemoglobin titrated wirh increasing concentration of NaOH. In addition this communication presents data indicating (1) wide variability in the extent of Na^+ adsorption among different isolated proteins and even among different samples of the same protein. (2) auto-cooperativity in the pH titration and Na^+ adsorption, and (3) selectivity of the liberated fixed anionic groups for different alkali-metal ions in the rank order Na* > Li > K > Rb, Cs.

INTRODUCTION

The membrane theory has dominated the biological sciences for nearly a century. It has been widely taught in textbooks and has served as the foundation for much of biomedical research. In this theory, intracellular ions (primarily K^+) and the bulk of cell water exist in a free state. The preferential accumulation of K^+ in the cell over Na^+ was first explained as due to an impermeability of the cell membrane to both K^+ and Nd. This explanation gave way to one in which the membrane was permeable only to K^+ but not to Na^+. This explanation was in turn replaced by the concept that the cell membrane is permeable to both K^+ and Na^+, and that asymmetry in their distribution is maintained by membrane pumps at the expense of continual energy expenditure (for history, see Ling, 1984).

The broad acceptance of the membrane-pump theory was based on what seemed to be good supportive evidence in its favor, and on the apparent failure then to produce indisputably convincing evidence for an alternative theory. Thus the possibility that the selective accumulation of K^+ over Na^+ in living cells might represent selective K^+ binding or adsorption on intracellular proteins".' was rejected by Lillie (1923) because earlier efforts to demonstrate alkali-metal ion binding on isolated proteins produced negative results.',"," Amongst these early reports of failure to show ion binding in *vitro* was one exception; the brief but widely known announcement of a successful demonstration of selective K^+ binding over Na^+ by isolated myosin.[26] Later failures to confirm this finding[33,7] further strengthened the view that proteins do not bind alkali metal ions selectively, nor to a high enough level necessary to explain the selective K^+ accumulation in living cells. However, the K^+ binding theory was not the only theory that seemed to be at odds with experimental findings.

From the early fifties on, a new kind of experimental finding began to cast doubts on the membrane-pump theory. By definition, a continuous expenditure of energy is necessary to operate the postulated membrane pumps. The energy supply of a living cell is

not unlimited. However, as time went on, more and more pumps were postulated. By 1968 Ling et *al.* already counted well over 20.[18] Yet under controlled conditions, one pump (the Na pump) alone, was shown to consume at least 15 to 30 *times as* much energy as all the energy the frog muscle cells commanded.[10] In the thirty years following, this work has not been challenged. Nor has anyone given even a hint just how an energy balance could be managed for the ever lengthening list of pumps both at the cell surface and at the even larger surfaces of the subcellular particles. While energy consideration was one of the earliest reasons that led Ling to reject the membrane pump concept, it was by no means the only reason. For a complete review of the whole story see Ling's *new* book, *In Search of the Physical Basis of Life.*[16]

In 1952 Ling suggested an electrostatic mechanism for the selective accumulation of K^+ in living cells.[9] In this theory the bulk of cell K^+ is preferentially adsorbed on the β- and γ-carboxyl groups of cellular proteins. As a result of what later Monod called an "allosteric" effect,[24] ATP acts as a "cardinal adsorbent" when it occupies key sites, called cardinal sites on proteins. Consequently, the β- and y-carboxyl groups of these proteins assume an electronic conformation or c-value* such that they preferentially adsorb K^+ over both free Na^+ and fixed cationic groups. The fixed cationic groups comprise primarily a-amino groups, c-amino groups, and guanidyl groups of the same or other proteins.[9,10] This theory, as a part of the much broader one, called the association-induction (AI) hypothesis, provides, on the one hand, a mechanism for selective K^+ accumulation that does not require a continual energy expenditure. Instead, it requires the presence of metabolically generated ATP per se. and not its hydrolysis for delivery of the so-called high energy. On the other hand, it provides an explanation for the failure to demonstrate selective alkali-metal ion adsorption on isolated proteins at neutral pH: In the isolated native proteins, the fixed anionic sites are masked, and not available for K^+ adsorption, because they are joined to the fixed cation groups in what Speakman and Hirst called "salt linkages"."

Thus if the fixed anionic β- and γ-carboxyl groups are represented by f^-, and the fixed cationic c-amino and guanidyl groups by f^+, the role of ATP as "cardinal adsorbent" in converting non-K'-adsorbing proteins to proteins selectively adsorbing K^+ (as found in living cells) can be represented by the following simple equation:

$$ff + K^+ + A^- \xrightarrow[-A]{+ATP} f\ A^- + f^-K^+, \qquad (1)$$

where A^- is the prevailing free anion in the system (e.g., Cl-). f-K+ represents K^+ adsorbed on f. $f\ A^-$ represents A^- adsorbed on f^+. f^+f^- represents the salt linkages.

It was argued that proteins both *in vivo* and *in vitro* tend to exist at minimum energy configurations. In the AI hypothesis the stability of these configurations significantly depend on the number of salt linkages formed

*The c-value is a parameter representing the electron density of a negatively charged oxygen atom. It is a way to quantitatively simulate the aggregate effects of the remaining atoms of an oxyacid on the interaction of a hypothetical, prototypic, singly-charged oxygen atom with a cation. It is expressed as a displacement (in angstrom units) of the unit electric charge on the oxygen atom from its original prototypic location at the center of the oxygen atom. Thus, if the aggregate effect produces an overall displacement of electrons in the system toward the oxyacid oxygen, it can hc exactly matched by a specific displacement of the unit charge toward the cation, represented as a positive c-value, e.g., + 1.0 A. On the other hand, if the aggregate effect is to produce the opposite effect. it would bc represented as a negative c-value, e.g., -1.0 A. Thus, a high c-value corresponds to a high pK_a, as in acetic acid, and a low c-value corresponds to a low pK_a, as in trichloracetic acid.

within each protein molecule and among different protein molecules.[9,10] During the historical elucidation of the three-dimensional hemoglobin structure by Perutz and his coworkers" and in years following, x-ray diffraction and other studies have dispelled any doubt that salt linkages are indeed part and parcel of the protein structure and that they enhance stability of isolated proteins as well as living cells comprising these proteins.[28]

According to the AI hypothesis, specific adsorption of alkali metal ions on cell proteins underlies a basic molecular mechanism which the living cell utilizes in discharging its varied physiological functions. In this mechanism, adsorption is one phase of an adsorptiondesorption cycle. The shift from one phase to the others is under the control of cardinal adsorbents like ATP and Ca^{++}.[16] In this transition, many alkali-metal binding anionic sites change in unison, made possible by a propagated short-range inductive effect between nearest neighboring sites. This type of falling domino-like, propagated inductive effect may give rise to *cooperativity* in the alkali-metal adsorption.

In the case of Na^+ competing against fixed cations for fixed anionic adsorption sites, the concentration of adsorbed Na^+ in moles per unit weight of the protein may be represented by the cooperative adsorption isotherm described by Yang and Ling:[11]

$$[Na^+]_{ad} = \frac{[f^-]}{2}\left\{1 - \frac{\xi - 1}{\sqrt{(\xi-1)^2 + 4\xi\theta}}\right\}, \qquad (2)$$

where

$$\xi = \frac{[Na^+]_{ex}}{[f^+]} \cdot K^{oo}_{f^+-Na^+}, \qquad (3)$$

and

$$\theta = \gamma/RT. \qquad (4)$$

$[f^-]$ and $[f']$ are the concentrations of fixed anionic and cationic sites in moles per unit weight of the protein. $[Na^+]_{ex}$ is the concentration of Na' in the surrounding medium. $K^{oo}_{f^+ \rightarrow Na^+}$ is the intrinsic equilibrium constant in the exchange of f^+ for Na^+ on the anionic sites. -γ/2 is the energy of nearest neighbor interaction. When -γ/2 is equal to 0, the adsorption shows no cooperative interaction among the adsorption sites; Equation 2 then reduces to the standard Langmuir adsorption isotherm. When -γ/2 is a negative value, the adsorption is heterocooperative. When $-\gamma/2$ is a positive value, the reaction is *autocooperative;* in this case, the adsorption of one ion (or solute) favors adsorption of more ions of the same species. A well known example of such an autocooperative binding is the binding of oxygen by hemoglobin. In linear plots of oxygen uptake against oxygen concentration in the medium, the curve is sigmoidal.

Equation 2 may be written in a simpler form, when $[f']$ is not specified. In that case, Equation 3 can be written as

$$\xi = \frac{[Na^+]_{ex}}{([f^-]-[Na^+]_{ad})} \cdot K^{oo}_{Na^+}, \qquad (5)$$

where $([f^-]-[Na^+]_{ad})$ is the concentration of anionic sites not adsorbing Na^+. $K^{oo}_{Na^+}$, like $K^{oo}_{f^+-Na^+}$ remains as an exchange constant, a pure number, and is not a true adsorption constant which is in units of M^{-1}.

Equation 2 can also be written in the logarithmic form:

$$\ln \frac{X_{Na}}{1 - X_{Na}} = \frac{[(\xi-1)^2 + 4\xi\theta]^{1/2} + \xi - 1}{[(\xi-1)^2 + 4\xi\theta]^{1/2} - \xi + 1} \qquad (6)$$

Analytical methods have been presented for testing for the existence and nature of cooperativity in an adsorption phenomenon:[14] (i) in a log-log plot of $X_{Na}/(1-X_{Na})$ *vs*

Na^+_{ex}, as in Equation 6, as well as in the simpler empirical Hill equation,[6] the slope of the curve at $X_{Na} = (1-X_{Na})$ yields the Hill coefficient n, long suspected to have some relation to the cooperativity. In fact n equals $\exp(-\gamma/2RT)$.[11] For autocooperative adsorption n exceeds the value of unity and $-\gamma/2 > 0$; (ii) in a Scatchard plot, the ratio of the concentration of adsorbed Na^+ over that of free Na^+ is plotted as the ordinate against the concentration of adsorbed Na^+ as abscissa.[30] The plot is convex upward when $-\gamma/2 > 0$ in an autocooperative adsorption; it is a straight line when $-\gamma/2 = 0$ as in a (non-cooperative) Langmuir isotherm.'," It has been shown that salt linkage dissociation represents a key step in the swelling of normal living cells in high concentrations of KCl and other alkali-metal halides[20,13] as well as in the swelling of injured cells in Na^+-containing medium." In the KCl-induced swelling of normal muscles, at least, the

Protein		pH	$[Na^+]_{total}$ (mM)	$[Na^+]_{free}$ (mM)	$[Na^+]_{bound}$	
Name	Concentration (%)				%	μmoles/100 g. dry protein
Bovine serum	15.6	5.06	209	201	3.83	5.13
	15.4	5.06	215	201	6.73	9.41
	14.1	5.14	219	208	4.85	7.50
	14.2	5.14	215	205	4.78	7.27
	(14.8±0.39)	(5.10±0.02)	(214±2.0)	(204±1.7)	(5.05±0.61)	(7.33±0.87)
Egg albumin	13.4	8.43	228	188	17.5	29.7
	13.5	8.47	233	193	17.2	29.5
	11.9	7.99	227	199	12.3	23.5
	12.0	7.99	227	199	12.4	23.5
	(12.7±0.43)	(8.22±0.13)	(229±1.4)	(195±2.6)	(14.85±1.44)	(26.35±1.76)
γ-globulin	18.9	6.61	217	196	9.76	11.22
	19.0	6.62	208	200	3.94	2.07
	15.6	6.64	215	205	4.55	6.27
	15.8	6.64	206	205	5.82	3.67
	(17.3±0.94)	(6.63±0.01)	(2112.6)	(201±2.2)	(6.02±1.3.)	(5.81±2.00)
Hemoglobin	16.6	7.47	203	198	2.6"	3.18
	16.6	7.47	203	200	1.61	2.04
	13.1	7.44	200	200	0.00	0.0"
	13.2	1.45	205	205	0.0"	0.00
	(14.9±0.99)	(7.46±0.01)	(203±1.0)	(201±1.5)	(1.07±0.64)	(1.30±0.79)
Myoglobin	13.8	7.49	209	194	7.44	11.27
	13.8	7.51	210	197	6.23	9.46
	11.0	7.41	208	198	5.02	9.5"
	11.4	7.43	209	197	5.97	10.96
	(12.5±0.75)	(746±0.02)	(209±0.41)	(197±0.86)	(6.16±1.24)	(10.30±0.48)

TABLE I. Na^+ binding on native globular protein at neutral pH. Hemoglobin, bovine serum albumin, ovalbumin, y-globulin, and hemoglobin were dissolved in 200 mM NaCl at a protein concentration of 20% (w/v) and then dialyzed against 200 mM NaCl at 4°C for I to 4 days.

reaction is highly autocooperative.

According to the "salt-linkage hypothesis" described by Equation 1 the fixed anionic groups will be liberated from the salt linkages and thus made available for alkali-metal ion adsorption if the fixed cationic groups are neutralized by removing their cationic charges. Suggestive evidence in support of this view could be found already in the 1935 publication of Fischer and Suer mentioned above.

In the case of a salt linkage formed between, say, a β-carboxyl group and an ε-amino group, the addition of NaOH until the pH reaches a value higher than the pK_a of the r-amino group (ca. 11 .0) would, according to the present theory, lead to Na^+ adsorption according to the following reaction:

$$R - \underbrace{COO^- \cdot NH_3^+}_{\text{salt linkage}} - R' + Na^+ + OH^- \rightarrow RCOO^- Na^+ + R'NH_2 + H_2O \quad (7)$$

where $RCOO^- Na^+$ represents Na^+ adsorbed on the unmasked β-carboxyl group with the disengagement of the e-amino group from the salt linkages following its neutralization.

This communication reports results of experiments designed to test this prediction of the salt-linkage hypothesis.

MATERIALS AND METHODS

All chemicals used were of reagent grade. The main protein studied was bovine hemoglobin obtained as a 2X crystallized, dialyzed, and lyophilyzed preparation from Sigma Chemical Company, St. Louis, Mo. mostly in the form of oxy- and methemoglobin (Lots 63F9324 and 100F9365). Other proteins studied were y-globulin (Sigma, Cohn Fraction II, Lot 60F9316 and 14F0554); bovine serum alumin (Sigma, Fraction V, Lot 31F0117 and 92F0721); myoglobin (Sigma, from horse heart, Type III, Lot 61F7036 and 61F7037). Egg albumin was from Nutritional Biochemicals control 2893.

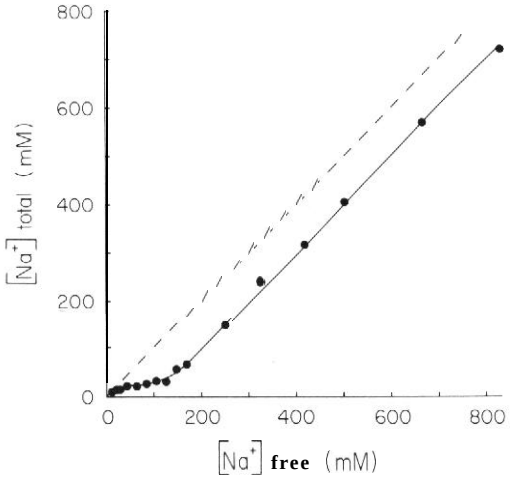

FIGURE 1. The relation between total Na^+ concentration and free Na^+ concentration (measured with the Na^+ selective electrode) in a 10% hemoglobin solution containing increasing amount of NaOH. Dashed line indicates the case where there is no adsorption and all Na^+ present is free.

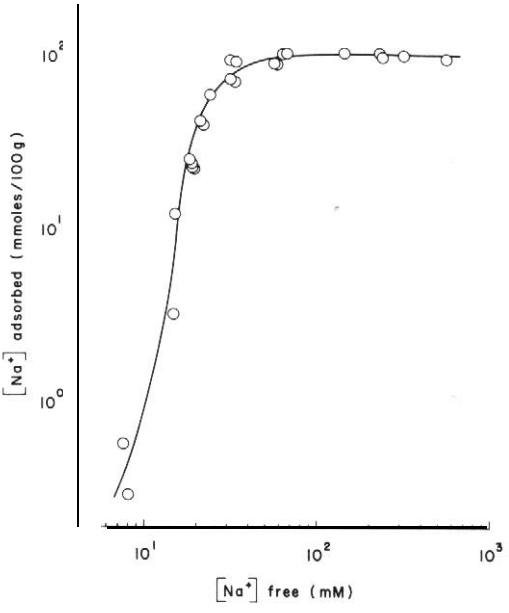

FIGURE 2. Logarithmic plot of the concentration of adsorbed Na^+ in 10% hemoglobin against free Na^+ in the medium. Data same as in Figure 1.

For pH measurements we used a wide-range glass electrode, calibrated for high pH measurement against a pH 12.0 ± .002 buffer standard (Hydrion). For free Na^+ concentration measurements we used a Corning 476-210 electrode in conjunction with a single junction calomel electrode (Model 90-01, Orion Res. Inc.). The Na^+/K^+ selectivity of this Na' electrode is 1000 at pH 7; the Na^+/Li^+ selectivity is 250. The care and use of this electrode were described in a preceding article.[21] The only point to be added is that although by themselves, K^+ and Li', for example, produce a potential reading 1000 or 250 times less effective than Na^+; the presence of K^+ and Li', especially at high concentration, significantly alters the calibration curves for Na^+. Therefore the standard curves must contain the same concentration of the co-existing ions and "sandwiching" of unknowns between pairs of standards of similar compositions are essential and routinely carried out.

To determine the total concentration of Na^+, by atomic absorption spectrophotometry, the protein-containing solutions were diluted to a final concentration of Na^+ in the range of 5 to 100 μM. A constant concentration of LiCl(97 mM) and of $NH_4H_2PO_4$ (3.0 mM) were present as radiation buffers in all the unknowns and standards.

RESULTS

Variable Na^+ Adsorption in Native Proteins at Neutral *pH.* Hemoglobin, bovine serum albumin, egg albumin, y-globulin, and myoglobin were dissolved in 200 mM NaCl at a 20% (w/v) concentration. The protein solutions were dialyzed against 200 mM NaCl at *4°C* for one to four days. The free Na'

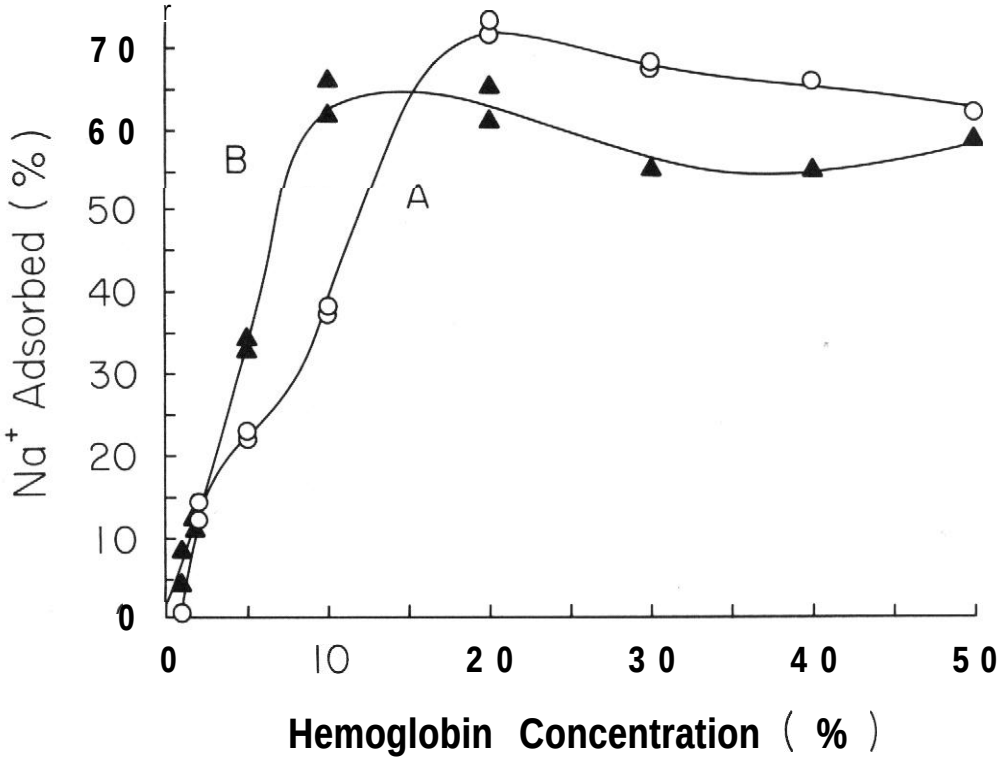

FIGURE 3. Concentration of adsorbed Na^+ in a 125 mM (A) or 250 mM (B) NaOH solution, containing increasing concentrations of hemoglobin.

concentrations of these solutions were measured with the Corning 476-210 Na electrode and compared with the total Na^+ concentrations determined by atomic absorption spectrophotometry (Table I). The difference, represented as a fraction of the total Na^+ present and referred to as bound Na^+, is given both in percentage and in units of μmoles of Na^+ bound to 100 grams of dry protein in Table I. (For evidence that the Na^+ invisible to the Na electrode is indeed adsorbed or bound, see below.) There is variation in the amount bound among different proteins. The overall picture is somewhat different from the view of scanty alkali-metal ion binding on native proteins referred to in the introductory section of this paper.

Proteins	Bound Na^+ (% of total Na^+)		
	1	2	3
Hemoglobin	53.7, 40.7, 50.4		
Bovine serum albumin	43.5, 36.8, 49.0	52.4	56.0
Egg albumin	40.4 57.8	45.0	34.0
γ-globulin	41.3 48.7	36.0	41.0
Myoglobin	46.2		

TABLE It. Percentage of bound fraction of Na^+ in NaOH-treated globular proteins. I, 20% protein in 200 mM NaOH kept at room temperature overnight. 2, 10% ;protein in 200 mM NaOH kept at room temperature then dialysis against 10 mM NaOH. 3. 10% protein in 400 mM NaOH kept at room temperature overnight then dialysis against 10 mM NaOH.

Consistently Higher Na^+ Adsorption on Proteins at High pH. Figure 1 plots the free Na^+ concentration measured with the glass electrode against the total Na^+ concentration measured by atomic absorption spectrophotometry in solutions of 10% hemoglobin in the presence of increasing concentrations of NaOH. The dashed line indicates the case where all Na'exists in the free state. Thus the difference between the solid and dashed lines indicates the concentration of Na^+ invisible to the Na electrode, presumably adsorbed. Evidence that this "invisible" Na^+ is indeed adsorbed on the protein will be presented in a following section.

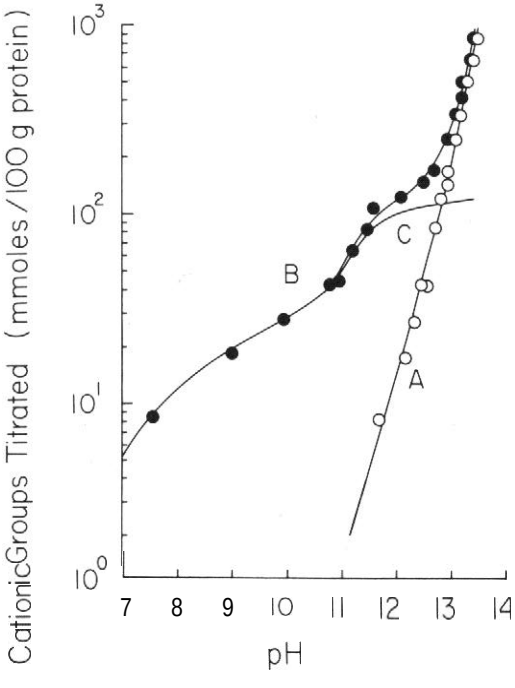

FIGURE 4. Titration curve of 10% hemoglobin (B) and of distilled water (A). The corrected titration Curve C, which was obtained by subtracting Curve A from Curve B, represents OH groups "bound" to hemoglobin at the different pH's.

In order to give better definition of the relation between free Na^+ and adsorbed Na^+ especially in the lower concentration range the data of adsorbed Na^+ is presented in a log-log plot in Figure 2. Note that the adsorbed Na^+ steadily increased from less than 1 mmoles per 100 g. of hemoglobin at a free Na^+ concentration of 7 mM until it reached a plateau with the adsorbed Na^+ concentration 105 mmoles per 100 g. of hemoglobin. Figure 3 shows the fraction of Na^+ adsorbed on increasing concentrations of hemoglobin added to a fixed concentration of NaOH (A: 125 mM; B: 250 mM).

Table II shows the percentage of adsorbed Na^+ measured when 10% or 20% bovine serum albumin, egg albumin, y-globulin, and

myoglobin bad been exposed to NaOH. In response, all of them exhibit large fractions of adsorbed Na^+ like hemoglobin. Thus the adsorption of Na^+ at high pH is not specific to hemoglobin but is a property shared by all proteins studied, in agreement with theory.

The Titration Curve of Fixed Cations in Hemoglobin. Figure 4 shows a titration curve of 10% hemoglobin (Curve B) and of the pH's of distilled water to which an increasing amount of NaOH had been added (Curve A). Curve A thus represents the free OH^- concentration at each of the pH's measured. Subtracting Curve A from Curve B, one obtains the "OH-binding" curve of hemoglobin (Curve C). At the highest pH measured (13.4), the total "bound" OH- was between 110 mM and 120 mmoles in one liter of the 10% hemoglobin solution. The data can also be expressed as 110 to 120 mmoles per 100 gram of hemoglobin. The binding of OH^- leads to the formation of water; its true significance in this paper lay in the fixed cation thus neutralized in consequence.

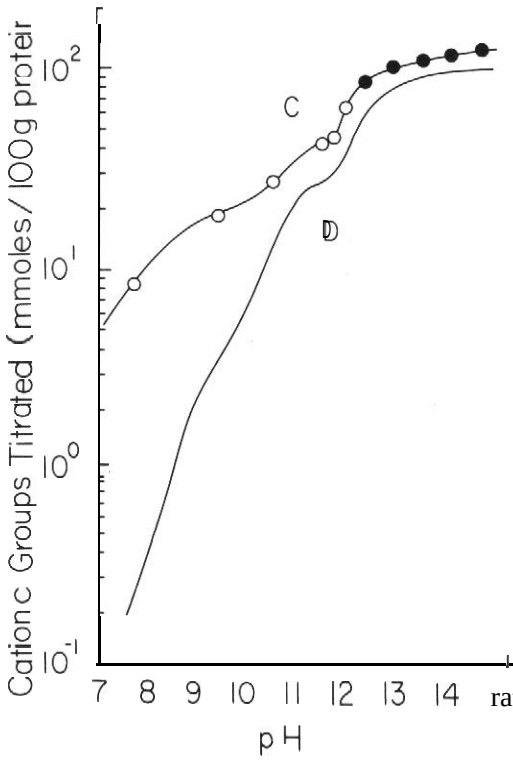

FIGURE 5. Comparison of theoretically calculated titration curve of hemoglobin (C) with experimental data. Experimental points were obtained from the data shown in Figure 4. The theoretically calculated curve is a composite of five calculated curves shown in Figure 6, corresponding to the titration of histidinc groups, α-amino groups, r-amino groups (2 types), and guanidyl groups. Curve D is the composite Curve C minus the histidinc group titration curve.

The data represented by Curve C of Figure 4 are represented once again in Figure 5. Here the six empty circles were those already shown in Figure 4 and transplanted from Figure 4 directly without alteration; five other points (solid circles) at pH equal to or higher than 11.5 were estimated from the corrected Curve C of Figure 4.

It is known that each hemoglobin molecule comprises two a-chains and two β-chains. Together they contain the following fixed cationic groups: 38 histidine groups, 4 a-amino groups, 44 s-amino groups, and 12 guanidyl groups.[29,34]

An examination of Curve C of Figure 4 reveals that the curve is not a smooth one but shows distinct humps. These humps indicate that a linear combination of Henderson-Hasselbach types of acid-base reactions or Langmuir adsorption isotherms will not fit the data. However, by using a family of isotherms described by Equation 2, we were able to produce a composite curve shown its the solid line of Curve C, which fits the data. (Note that we represented the hemoglobin in units of 100 grams of dry protein weight ratherthanits molecularweight of 67,000").

To obtain the theoretical curve shown. the following assumptions were made: Of the 38 histidine groups present in hemoglobin 16 mmoles/ 100 gram are titrated in this range of pH 7 to 13.5) and they all are assumed to have a pK_a of 7.5. In addition, a-amino groups, e-amino groups, and guanidyl groups which dissociate at higher pH's were all taken into account. The concentrations in units of

mmoles/100 grams are 6.66 for a-amino groups, and 20.6 for guanidyl groups. All α-amino groups, are given a pK of 9.0; all guanidyl groups a pK of 12.0. However, of the 66 mmoles/100 grams r-amino groups, 19 mmoles/100 grams were given a pK_a value of 10.0 and the remaining 47 mmoles/100 grams, a pK_a of 11.3. While the histidyl and. a-amino groups, were assumed to show no cooperative interaction, in order to tit the experimental data the titration of all the ε-amino groups and the guanidyl groups were assumed to be autocooperative. The theoretical titration curves of each of these individual components are displayed in Figure 6.

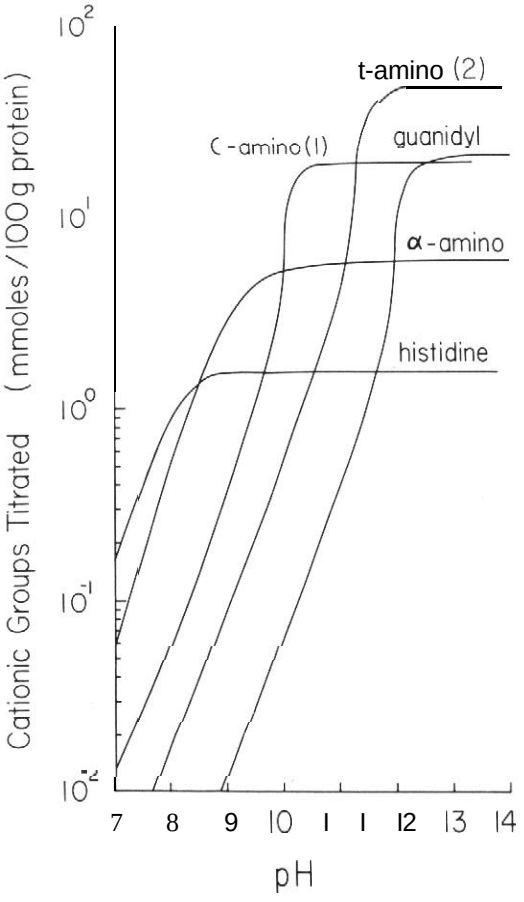

FIGURE 6. Individual titration curves of the histidine groups, n-amino groups, two types of ε-amino groups, and guanidyl groups of hemoglobin. For the pK_a values and concentrations see text. $-\gamma/2$ was 0 for u-amino groups and histidine groups but equal to 0.824 Kcal/mole for both types of r-amino groups and guanidyl groups.

The Relation of Na^+ Adsorption to the Titration of Fixed Cationic Groups. Since all the histidine groups are readily titratable and thus not masked in native hemoglobin in the neutral pH range,",' one may safely conclude that most histidine groups are not involved in salt-linkage formation.

As mentioned above, each molecule of hemoglobin contains 44 c-amino groups, 12 guanidyl groups, and 4 a-amino groups totalling 60 cationic groups. Each hemoglobin molecule also contains 34 β-carboxyl groups, 26 y-carboxyl groups, and 4 α-carboxyl groups, totalling 64. Thus, theoretically, there are more than enough fixed

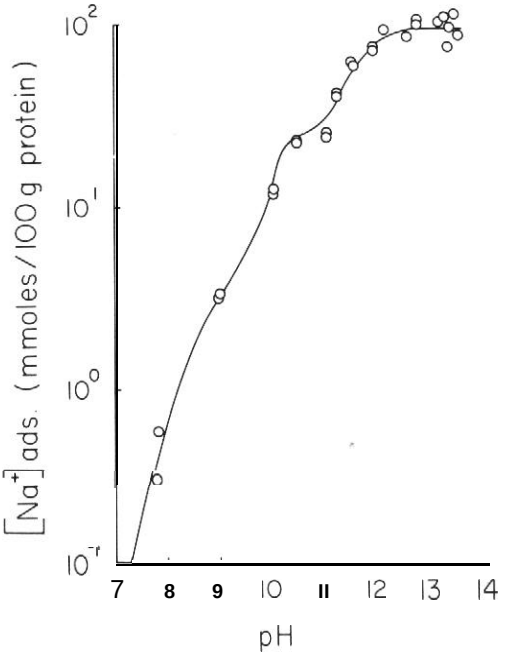

FIGURE 7. The quantitative relation between fixed cations neutralized and Na^+ adsorbed. Points are experimentally measured concentrations of Na^+ adsorbed on 10% hemoglobin at different pH's. The solid line going through or near most of the experimental points is the same theoretically calculated Curve D (of Figure 5), which is a composite of the titration or neutralization curves of all the u-amino groups, e-amino groups, and guanidyl groups in 100 g. of hemoglobin.

anionic carboxyl groups to form salt linkages with all the a-amino groups, e-amino groups, and guanidyl groups present in a hemoglobin molecule.

Let us now assume that in the oxyhemoglobin-methemoglobin mixture we studied, all the 60 *non-histidine cationic groups are* locked in salt linkages with β- and γ-carboxyl groups. Let us further assume that every anionic a-, β-, or y-carboxyl group liberated from the salt linkages after titration and neutralization of the cationic groups binds one Nd. With these assumptions, one can predict that for each mole of cationic groups neutralized, one mole of Na^+ will be adsorbed.

Now we have already shown that the titration curve of hemoglobin (Figures 4 and 5) in the pH range from 7.0 to 13.5 is adequately described by the combination of the five individual titration curves shown in Figure 6. Therefore we can readily obtain the total profile of the titration of *non-histidyl fixed cationic* groups by simply subtracting from Curve C of Figure 5, the histidine titration curve shown in Figure 6. The result is shown as Curve D of Figure 5. One wants to know if the Na^+ adsorption curve resembles this Curve D.

We calculated the concentration of adsorbed Na^+ in hemoglobin solutions to which

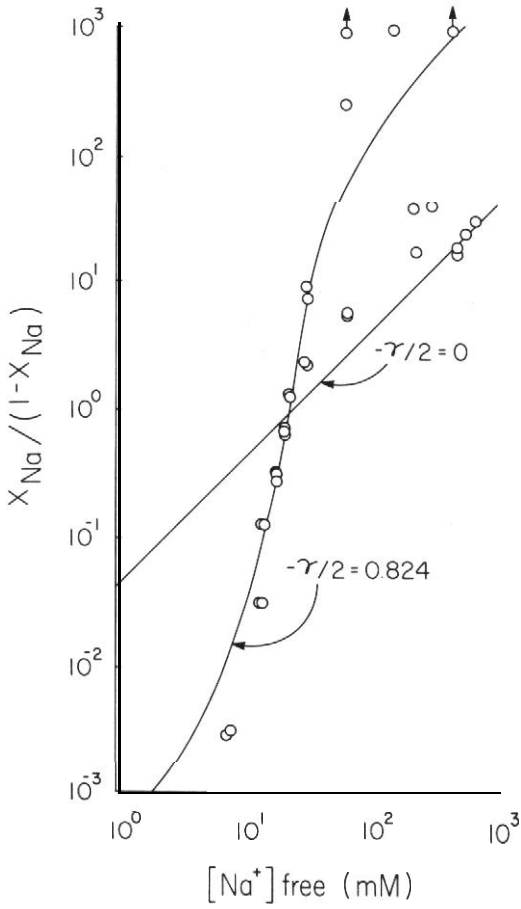

FIGURE 8. Log-log plot of Na^+ adsorbed on 10% hemoglobin against the concentration of free Na^+ in the medium. Solid line is a theoretical curve calculated according to Equation 2 with $K^{\infty}_{X \to Na}$ equal to 4.15×10^2 and $-\gamma/2 = 0.824$ Kcal/mole (n = 4.09). Straight line is a theoretical (Langmuir) adsorption curve with -y/2 = 0 Kcal/mole (n = I).

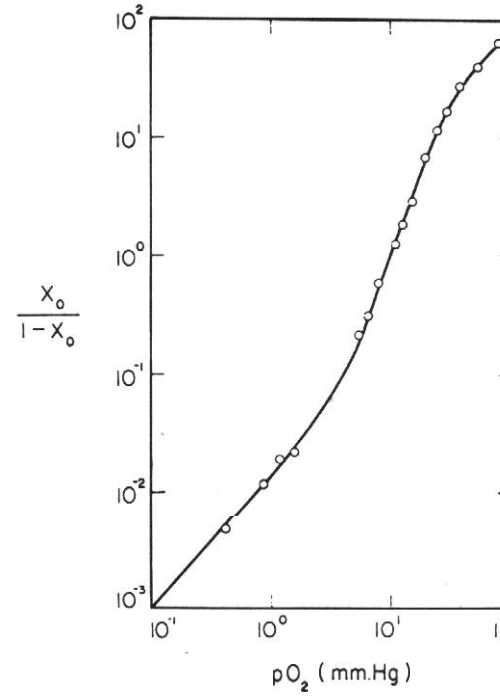

FIGURE 9. A lag-log plot of the data of Lyster on oxygen uptake by human hemoglobin at pH 7.0 at 19°C. Points are experimental: the line is theoretical with K = 0.1 (mm Hg)$^{-1}$ and $-\gamma/2$ = 0.67 Kcal/mole (data of Lyster, cited in Rossi-Fanelli et a,., ,964. From Ling (1969) by permission of Intern. Rev. Cytology).

increasing concentrations of NaOH were added. The adsorbed Na^+ concentration was obtained by subtracting, from the total Na^+ determined by atomic absorption spectrophotometry, the free Na^+ concentration measured with the Na^+ specific glass electrode. The data calculated are shown as empty circles in Figure 7 and are plotted against the final pH's of the solutions.

The solid line which stays close to most of the experimental data points is in fact exactly the some *as* Curve *D of Figure* 5. This quantitative concordance of Na^+ adsorption and cationic group neutralization verifies Equation 7 as well as the two assumptions which underlied this prediction: (1) all the non-histidyl cationic groups, including all the u-amino groups, all the ε-amino groups, and all the guanidyl groups of the native hemoglobin molecules are locked in salt linkages with β- and y-carboxyl groups in the native state at neutral pH. (2) each β- and γ-carboxyl group liberated adsorbs one Nd.

Autocooperativity in the Adsorption of Na^+ by Hemoglobin at High pH's. In Figure 8, we have plotted the data of Na^+ adsorption on hemoglobin according to Equation 6. The best-fitting curve was based on a $K^{oo}_{f+\to Na+}$ equal to 24 and -y/2 equal to 0.824 Kcal/mole. The straight line is the theoretical curve also calculated according to Equation 6 in which there is no site-to-site nearest neighbor interaction energy (-y/2 = 0). Although the scattering of data at high Na^+ concentration is considerable, the data as a whole clearly indicate a high degree of autocooperative interaction among the Na^+ adsorption sites, as is the case among the 4 heme sites in the oxygen binding on hemoglobin similarly analyzed as shown in Figure 9.[22,12] The Scatchard plot (Figure 10) shows a curve with an

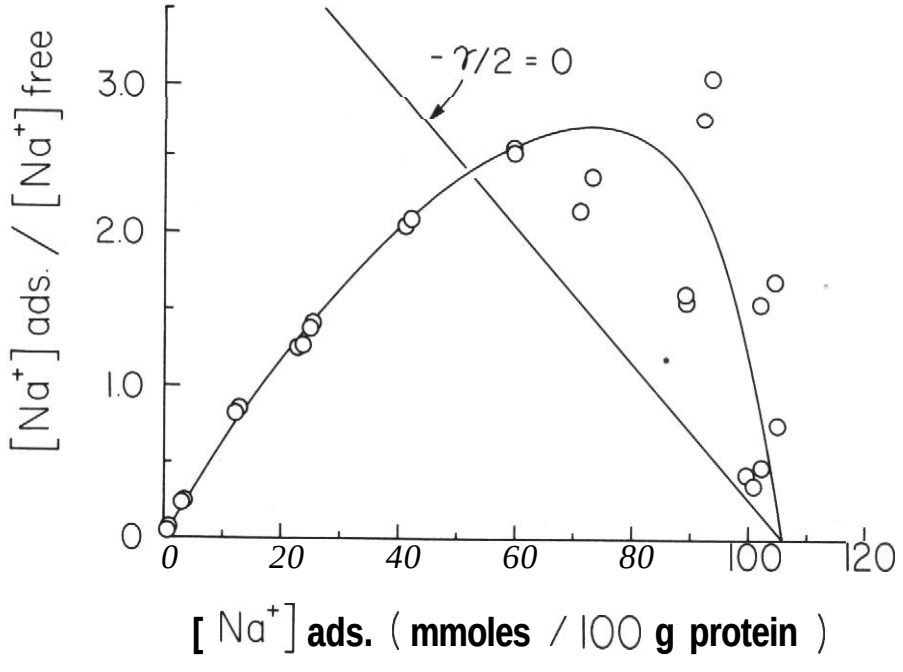

FIGURE 10. Scatchard plot of the adsorption of Na^+ on 10% hemoglobin. Curve shows upward convexity typical of autocooperative adsorption. Straight line shows a theoretical (Langmuir) adsorption isotherm ($-\gamma/2 = 0$ Kcal/mole).

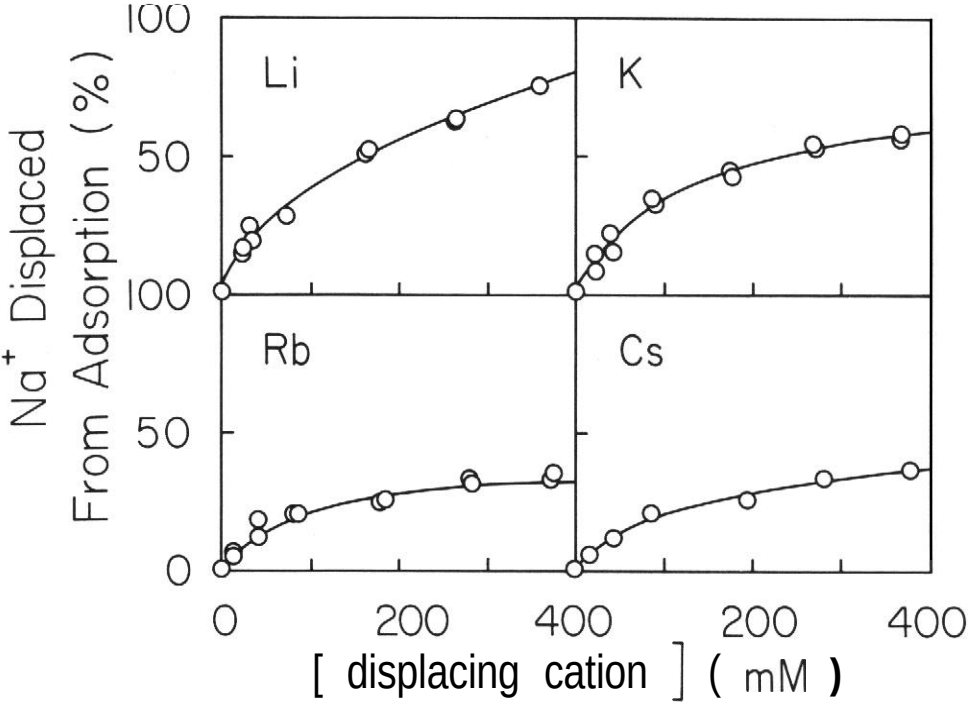

FIGURE 11. Displacement of adsorbed Na^+ by Li', K^+, Rb^+, and Cs^+. Ordinate represents percentage of adsorbed Na^+ displaced by different concentrations of the displacing ions, Li^+, K^+, Rb^+, or Cs^+ all added as chlorides in a 10% solution of hemoglobin containing a fixed concentration of NaOH (125 mM).

upward convexity typical of autocooperative binding. If there were no autocooperative interaction, the points should fall on a straight line, as indicated by the straight line marked with the sign, $-\gamma = 0$.

Specificity in Alkali-metal Ion Adsorption. Hemoglobin, initially at 10% (w/v), was dissolved in 125 mM NaOH and then incubated in solution containing NaOH at a constant level (125 mM) and varying concentrations of LiCl, KCl, RbCl, CsCl. The free and adsorbed Na^+ concentrations were determined. From the increase of free Na^+ measured, one calculates the percentage displacement of adsorbed Na' by Li', K', or Cs', as well as the free and bound Li^+, K', Rb', or Cs' as the case may be. The data obtained are plotted in Figure 11 as a function of the concentration of free Li', K', Rb', or Cs' in the final incubation solutions. Pronounced differences are seen in the effectiveness of the 4 alkali-metal ions in displacing Na^+, following the rank order $Li^+ > K^+ >$ Rb', Cs^+. Since each of these alkali-metal ions have the same long-range attributes (e.g., univalency) but different short-range attributes (e.g., polarizability, dipole moment, Born repulsion constants), the differences in their efectiveness in displacing Na^+ "invisible" to the Na specific electrode and making it "visible" could only be the result of competition for adsorption sites where the free cation and fixed anion are in close contact.[19,13,21] Otherwise, the differences among these alkali-metal ions would not be "felt," and therefore their displacing effectiveness would have been the same, contrary to observation.

In Figure 12 the same sets of data shown in Figure 11 are plotted in a different way. Here the ordinate represents the ratio of the mole fractions of sites adsorbing Li^+, K^+, Rb^+, or Cs' (X_{Li}, XK, X_{Rb}, Xc,) divided by the mole fraction of the sites adsorbing Na^+ (X_{Na}). The

abscissa represents the ratios of free Li^+, K^+, Rb', or Cs' over that of free Na^+ in the bathing solution at final equilibrium. Equation 6 shows that at equal occupancy (e.g., $X_{Li} = X_{Na}$, hence $X_{Li}/X_{Na} = 1$), the free ion concentration ratio (e.g., $[Li]_{free}/[Na^+]_{free}$) is equal to the reciprocal of the intrinsic equilibrium constant, $K^{oo}_{x^+ \to Na^+}$ where x stands for Li', K', Rb', or Cs': 2.3 (Na/Li); 3.6 (Na/K); 14.0 (Na/Cs) and 16.5 (Na/Rb). Since the Rb' and Cs' data did not extend beyond the equal occupancy point (i.e., X_{Rb} or $X_{Cs} = X_{Na}$), the data bad to be obtained by extrapolation. Thus while there is no question that the adsorption for Cs^+ and Rb' are the weakest among the five alkali-metal ions studied, it is not certain if Rb^+ is truly preferred over Cs^+.

The data of Figure 12 also permits an estimate of the quantitative parameters describing their cooperativity. For the Na/Li and Na/K pairs, n is equal to 1.25 and 1.09 and $-\gamma/2$ equal to $+0.13$ and $+0.05$ Kcal/mole respectively. The slopes of the Na/Rb and Na/Cs curves are both less than unity, with n equal to 0.72 and 0.67 for Na^+/Cs^+ and Na^+/Rb^+ respectively. Low n-values may be due to true heterocooperativity or due to heterogeneity in the binding sites. The data presented here are not complete enough to offer a definite choice.

DISCUSSION

The Identity of the Alkali-metal Ion Binding Groups in Hemoglobin. That one can predict from the known concentrations of cationic polar side chains of hemoglobin and reasonable pK_a values, the measured titration curve of hemoglobin is not surprising. Similar matching of titration data on proteins including hemoglobin have been reported before." However, it is remarkable that this same calculated titration curve can also fit the Na^+ binding data. This finding is new and would be very difficult to explain if one assumes that all cationic and anionic side chains in hemoglobin were free to begin with.

The concordance of the Na^+ binding curve and the titration curve becomes readily understandable if we assume that these cation& groups were originally joined to the fixed anionic groups which were thus unable to bind Na^+, as the data of Table I has shown. However, once the competing cationic groups are put out of function by their neutralization, the carboxyl groups become liberated. The fitting of the Na^+ binding data by the titration curve shows that the reaction described by Equation 7 proceeds fully to the right. With each cationic group neutralized, there immediately followed one Na^+ adsorbed.

The present findings have dispelled an age-old myth that alkali-metal ions do not significantly adsorb on proteins. This myth was in

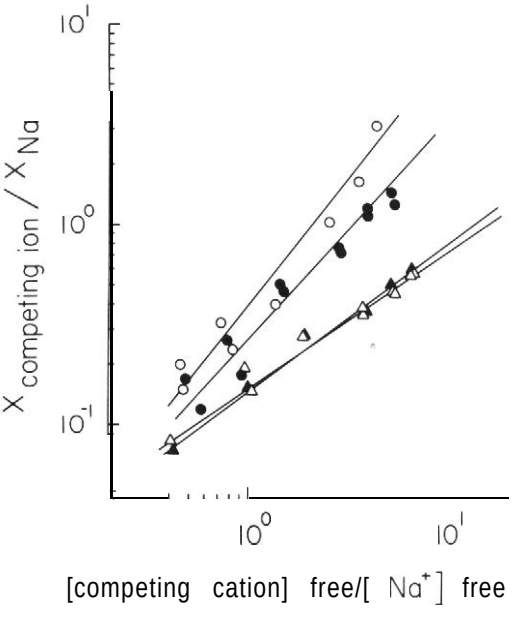

FIGURE 12. Lag-log plot of the concentration ratio of adsorbed competing ion over that of adsorbed Na^+, against the concentration ratio of free competing ion over that of free Na^+. $X_{competing\ ion}$ and X_{Na^+} are the mole fractions of sites adsorbing the competing ion or Na^+. Intercept at $X_{competing\ ion}/X_{Na} = 1$ is equal to $K^{oo}_{x^+ - Na^+}$; Na/Li = 2.3; Na/K = 3.6; Na/Rb = 16.5; Na/Cs = 14.0.

part the result of the early failure to demonstrate alkali-metal ion binding on proteins, already mentioned, and in part the result of unjustified extensions of the theory and experimental data on the scanty alkali-metal ion association with free anions (e.g., Cl^-) in dilute solutions. Ample theoretical reasons were given, why association of counterions greatly increases when one species of the interacting ion is fixed in macromolecular chains.[10,16] Thus with the removal of the competing fixed cations, proteins bind alkali-metal ions to high degrees much as polystyrene sulfonate[21] and polymethacrylate do (Kern, 1948).

The findings described in this paper also agree well with the important findings of Steinhardt and Zaiser (1955) who titrated hemoglobin with acids. They found that 36 groups were not accessible for titration in native hemoglobin but were explosively released in a" all-or-none manner when the pH decreased to a critical value between 3.1 and 3.5. From their pK_a's one calculates that these groups released must be primarily carboxyl groups.

The reason offered by Steinhardt and Zaiser (1955) for the masked condition of the carboxyl groups, and reviewed by Edsall and Wyman (1958, p. 539), is that they are joined in salt linkages with ϵ-amino groups of lysine residues, They offered a set of convincing arguments in support of this view. The isoelectric point (IEP) of hemoglobin remains unchanged "ear pH 7 before and after denaturation, which signifies that the liberation of anionic carboxyl groups during titration with acids could not have occurred alone. Otherwise, the acid denatured hemoglobin would have a much lower IEP than the native hemoglobin. The constancy of IEP therefore requires that with the liberation of each carboxyl group, there is also liberated one basic group. This follows naturally if masking of both groups in the native protein is due to their being engaged with each other in the one-to-one stoichiometry of a salt linkage. Our present findings in combination with Steinhardt and Zaiser's data have shown that both alkali and acid can liberate at once both the acidic groups and the basic groups. However, our present findings have shown in addition that the basic groups involved are not limited to the c-amino groups of lysine, as Steinhardt and Zaiser's studies indicated, but include the a-amino groups and guanidyl groups as well — all of which must be masked in the native hemoglobin by joining with a" equal number of a-, β-, and γ-carboxyl groups.

The Molecular Mechanism of the Autocooperativity in Ion Binding. The sigmoid-shaped uptake curve of oxygen on hemoglobin has been employed to describe the quantitative aspects of the phenomenon. A coherent interpretation of this type of behavior came late into the picture. Thus in 1964 Ling presented what has been known as the Yang-Ling isotherm, shown earlier as Equation 2, and demonstrated the usefulness of this theory in terms of the association-induction model." The work of Monod and his colleagues on what has been called allosteric enzymes enhanced the general interest in cooperative phenomena.[24]

In several earlier publications, Ling (1969, 1980, 1981, 1984) has pointed out that autocooperative interaction occurs not only among binding sites situated on different subunits (e.g., heme-heme interaction in hemoglobin) but also on sites on the same protein chains of the subunits (e.g., binding of cationic detergent, dodecyltrimethylammonium bromide to bovine serum albumin; phenol binding on collagen). Therefore at the most fundamental level, autocooperativity among binding sites does not require specific symmetry matching or other mechanism requiring the existence of subunits, although these and other factors may play a role in specific cases. Rather autocoopera-

tivity appears to be a basic property of all protein chains — in agreement with the AI hypothesis. In this hypothesis, the near neighbor interaction underlying this cooperative interaction has a major inductive component, and is mediated through the highly polarizable polypeptide chains possessed by all proteins. The present findings reveal once more that autocooperativity exists not only among heme sites of the four hemoglobin subunits, as in the binding of oxygen, but throughout each of the subunits since virtually all the cationic (except histidine group) and anionic carboxyl groups are involved and these groups are distributed throughout the two a and two β-chains of a hemoglobin molecule.

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