clusive, they tend to support the hypothesis of an equilibrium mixture of labile isomers,[15]

Experimental.—The spectra were determined on Perkin–Elmer model 12c spectrometers using sodium chloride prisms and carbon disulfide solutions. For the low temperature measurements in solution a cell similar to that described by Bernstein[16] was employed, and for measurements of cooled solid films a Hornig type cell was employed.[17]

For the evaluation of the integrated absorption intensities the curves were plotted as apparent optical density ($\log_{10}(T_0/T)_\nu$) against the frequency in wave numbers on mm. graph paper. The area under the curve between 1190 and 1290 cm.$^{-1}$ was computed by counting squares and the apparent integrated absorption intensity (B) evaluated from

$$B = \frac{e}{cl}\int_{1190}^{1290} \log_{10}\left(\frac{T_0}{T}\right)_\nu d\nu \text{ mole}^{-1}/\text{liter/cm.}^{-2}$$

the equation in which T_0 and T are the intensities of the incident and transmitted radiation when the spectrometer is set at a frequency ν, c is the concentration of the solute in moles per liter, l the cell length in cm. and e the base of the natural logarithms. The intensities were measured at a slit width of 5 cm.$^{-1}$.

Concluding Remarks.—The recognition of the principal *functional groups* in steroids by infrared spectrometry, and the correlation of the position of the group in the molecule with the frequency of certain absorption bands has been reported,[3,7,10] and recently summarized.[14] Although the suggestion has been made previously[18] that the *stereochemical configuration* of a steroid may be associated with characteristic absorption bands in the "fingerprint" region, the studies described here provide the first instance in which we have observed such a correlation to hold over a wide variety of compounds. The use of this correlation, in association with the digitonin precipitation reaction, for the unique assignment of the stereochemical configuration at positions 3 and 5 provides an additional example of the importance of considering spectrographic and chemical evidence together in the elucidation of molecular structure.

Acknowledgments.—The authors wish to thank the several investigators, listed individually in a footnote to Table I, who kindly made available many of the compounds. The technical assistance of Miss L. Groth, Mr. D. S. Keir and Mr. R. Lauzon at the National Research Council and Miss B. Boland and Miss R. Connolly at the Sloan–Kettering Institute is also gratefully acknowledged. This investigation was aided by grants from The American Cancer Society (upon recommendation of the Committee on Growth of the (U. S.) National Research Council), Ayerst, McKenna and Harrison, Ltd., the Jane Coffin Childs Memorial Fund for Medical Research, The Commonwealth Fund, the National Cancer Institute of the National Institutes for Medical Research, U. S. Public Health Service and the Anna Fuller Fund.

(15) In an attempt to achieve a greater temperature difference, the spectrum of a solid film of androsterone acetate was measured at room temperature and when cooled with liquid nitrogen. The curve, which is included in Fig. 5, indicated that at room temperature the acetate band system is less well resolved than in carbon disulfide solution. On cooling the acetate bands are not appreciably affected, although neighboring bands sharpen considerably.

(16) Powling and Bernstein, THIS JOURNAL, **73**, 1815 (1951). The assistance of Dr. Bernstein in making these measurements is gratefully acknowledged.

(17) Wagner and Hornig, *J. Chem. Phys.*, **18**, 296 (1950).

(18) Furchgott, Rosenkrantz and Shorr, *J. Biol. Chem.*, **163**, 375 (1946); *ibid.*, **167**, 627 (1947).

NEW YORK, N. Y.
OTTAWA, CANADA RECEIVED JANUARY 15, 1951

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF WISCONSIN]

Study of Protein–Ion Interaction by the Moving Boundary Method. The Combination of Bovine Serum Albumin with Chloride Ion[1]

BY ROBERT A. ALBERTY AND H. H. MARVIN, JR.[2]

The binding of chloride ions by crystallized bovine serum albumin in 0.15 molar sodium chloride at 0° has been determined by the moving boundary method. This method involves the accurate measurement of the chloride and sodium constituent mobilities in a protein solution and interpretation of the results in terms of the moving boundary equation for weak electrolytes. The measurements at *p*H 7.00, 5.40 and 3.20 indicate the binding of 8, 9 and 29 chloride ions per molecule of albumin, in excellent agreement with the values obtained by the membrane equilibrium and electromotive force methods.

Evidence for the binding of chloride ion by serum albumin has been obtained by a variety of physical measurements: these include osmotic pressure,[3] membrane equilibrium[4] electromotive force[4] and shift in isoelectric[5,6] and isoionic[7] points. In view of the importance of this particular interaction in determining the properties of serum albumin under physiological conditions we have undertaken a study of the binding of chloride ion by crystallized bovine serum albumin in 0.15 molar sodium chloride by the moving boundary method. This method has recently been described[8,9] and has been applied by Smith and Briggs[9] to the study of the interaction of bovine serum albumin with methyl orange. The method depends upon the measurement of the constituent mobility of the interacting ion and of the protein in a solution of known composition and is applica-

(1) This material is taken from the Ph.D. thesis of H. H. Marvin, Jr., University of Wisconsin, August, 1950.

(2) United States Rubber Company Fellow, 1949–1950. Research Laboratory, General Electric Co., Schenectady, N. Y.

(3) G. Scatchard, A. C. Batchelder and A. Brown, THIS JOURNAL, **68**, 2320 (1946).

(4) G. Scatchard, I. H. Scheinberg and S. H. Armstrong, Jr., *ibid.*, **72**, 535 (1950).

(5) L. G. Longsworth and C. F. Jacobsen, *J. Phys. Colloid Chem.*, **53**, 126 (1949).

(6) R. A. Alberty, *ibid.*, **53**, 114 (1949).

(7) G. Scatchard and E. S. Black, *ibid.*, **53**, 88 (1949).

(8) R. A. Alberty and H. H. Marvin, Jr., *ibid.*, **54**, 47 (1950).

(9) R. F. Smith and D. R. Briggs, *ibid.*, **54**, 33 (1950).

ble, in its present form, only if the rates of complex formation and dissociation are fast compared to the duration of the experiment. The mobilities of small ions in solutions of inorganic colloids have been studied by Bikermann[10] and the mobility of radiosodium in solutions of two anionic and two cationic surface active agents has been studied by Brady and Salley.[11] As indicated by these authors quantitative information concerning binding may be obtained.

Moving Boundary Systems.—The sodium and chloride ion mobilities in 0.15 molar sodium chloride at 0° were determined by means of the moving boundary systems[12] 0.07 *M* LiCl(γ)::LiCl(β) → 0.15 *M* NaCl(α) and 0.15 *M* NaCl(α) ← NaF(β)::0.07 *M* NaF(γ). The systems used to determine the sodium and chloride constituent mobilities are shown in Fig. 1. System (a) differs from that used to determine the sodium ion mobility only in that protein was initially present in the α solution. In the measurement of the chloride constituent mobility this simple arrangement of the experiment is not possible since the descending chloride constituent boundary in this case is gravitationally unstable. It is necessary to invert the system, using an indicator anion heavier than chloride and dissolving albumin in the indicator solution, as shown in Fig. 1(b) so that the chloride constituent boundary ascends into the albumin solution which is being studied. Dole's theory[13] was useful in calculating the concentrations to be used in the initial solutions to yield gravitationally stable-moving boundary systems. In accordance with Dole's theory there are two moving boundaries in addition to the $\alpha\beta$ concentration boundary, one of these ($\beta\gamma$) being of the type across which no ion disappears. If the protein is below its isoelectric point, the moving concentration boundary descends as shown in Fig. 1(c).

Since the sodium constituent disappears in the $\alpha\beta$ boundary of Fig. 1(a) the moving boundary equation[14] for this constituent reduces to

$$\bar{u}^{\alpha}_{Na} = v^{\alpha\beta}\kappa^{\alpha} \qquad (1)$$

where $\bar{u}^{\alpha}_{Na}$ is the sodium constituent mobility, $v^{\alpha\beta}$ is the volume moved through by the boundary in ml. per coulomb, and κ^{α} is the specific conductance of the α solution at 0°. Similarly, for systems (b) and (c) the chloride constituent mobility may be calculated from

$$\bar{u}^{\delta}_{Cl} = v^{\gamma\delta}\kappa^{\delta} \qquad (2)$$

Experimental

The sodium and chloride ion mobilities and their constituent mobilities in the presence of albumin were determined in the standard Tiselius cell with the aid of a cylindrical lens schlieren optical system. A photoelectric current regulator[15] was used, and the general procedure was essentially that described earlier.[16] The exposures were made

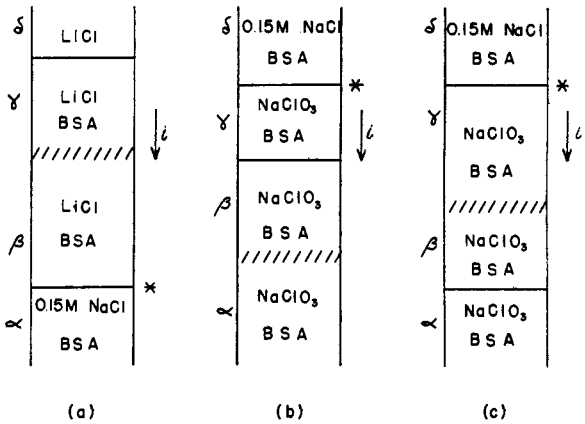

Fig. 1.—Moving boundary systems used for the determination of (a) $\bar{u}_{Na}$ at *p*H 7.0 and 5.4, (b) $\bar{u}_{Cl}$ at *p*H 7.0 and 5.4, (c) $\bar{u}_{Cl}$ at *p*H 3.20. The boundary yielding the desired constituent mobility is indicated by an *.

by an automatic camera[17] which was controlled by synchronous motors so that the interval between exposures was constant within a few tenths of a second. A Gaertner microcomparator was used to determine the distances moved by the boundaries.

The boundary velocities measured with respect to the cell were corrected for the movement of the solvent according to the usual procedures.[18] In the case of the mobilities determined in protein solutions it is necessary to add a term to take into account the migration of the protein.[19] Thus the correction for the sodium constituent boundary which moved into the albumin solution is

$$\Delta V = V_{Ag} - V_{AgCl} + T_{Na}\bar{V}_{NaCl} + \frac{\phi_p c_p \bar{u}_p\ 96.5}{\kappa_p}$$

where V represents molal volume, $\bar{V}$ partial molal volume, ϕ apparent specific volume, κ specific conductance, c concentration in g.l.$^{-1}$, T transference number and the subscript p refers to the protein.

The bovine serum albumin was obtained from Armour Laboratories, from lots (control no. 325 and 284-288) which were recrystallized in the spring of 1950. These samples have been found to be more homogeneous electrophoretically than older samples.[20] A concentrated solution of albumin in distilled water was prepared and dialyzed at 2° against three changes of distilled water to eliminate traces of foreign electrolytes. The protein concentration was determined by measuring the light absorption at 280 mμ with a Beckman spectrophotometer. The various solutions were prepared from stock solutions of the various salts and the *p*H was adjusted by the slow addition of dilute HCl, NaOH or $HClO_3$. The protein itself was the only buffer present. The *p*H was measured with a glass electrode at 25°.

Theory

If complexes of the type PCl, PCl_2, PCl_3, ..., PCl_n, where P represents the protein, are in equilibrium and the rates of formation and dissociation of these complexes are fast compared to the duration of a mobility determination, the system may be treated by the theory developed for solutions of weak electrolytes.[14] It has previously[8] been shown that quantitative information concerning the interaction may be obtained from the various constituent and ion mobilities if (I) a single complex is formed and the concentration of free P is negligible, (II) a Langmuir distribution of complexes with mobili-

(10) J. J. Bikermann, *Trans. Faraday Soc.*, **33**, 560 (1937).
(11) A. P. Brady and D. J. Salley, THIS JOURNAL, **70**, 914 (1948).
(12) L. G. Longsworth, *ibid.*, **67**, 1109 (1945).
(13) V. P. Dole, *ibid.*, **67**, 1119 (1945).
(14) H. Svensson, *Acta Chem. Scand.*, **2**, 855 (1948); R. A. Alberty and J. C. Nichol, THIS JOURNAL, **70**, 2297 (1948); R. A. Alberty, *ibid.*, **72**, 2361 (1950); J. C. Nichol, *ibid.*, **72**, 2367 (1950).
(15) N. E. Bonn, H. H. Marvin, Jr., and R. A. Alberty, *Rev. Sci. Instr.*, in press.
(16) R. A. Alberty and E. L. King, THIS JOURNAL, **73**, 517 (1951).
(17) R. M. Bock and E. Hansen and R. A. Alberty, in preparation.
(18) L. G. Longsworth, THIS JOURNAL, **54**, 2741 (1932); **65**, 1755 (1943).
(19) L. G. Longsworth and D. MacInnes, *ibid.*, **62**, 705 (1940).
(20) R. L. Baldwin, P. M. Laughton and R. A. Alberty, *J. Phys. Colloid Chem.*, **55**, 111 (1951).

ties $u_{PCl_i} = u_P + iw$ are formed (where w is a constant), or (III) a single complex is in equilibrium with both free protein and chloride ion. To these three cases will be added a case IV to be discussed below.

In case I the number of equivalents of chloride ion bound per mole of albumin, $\bar{v}_{Cl}$, may be calculated from

$$\bar{v}_{Cl} = \frac{[\bar{Cl}](\bar{u}_{Cl} - u_{Cl})}{[\bar{P}](\bar{u}_P - u_{Cl})} \quad (4)$$

where $[\bar{Cl}]$ is the total chloride concentration, $[\bar{P}]$ is the molar concentration of protein, u_{Cl} is the mobility of *free* chloride ions and $\bar{u}_{Cl}$ and $\bar{u}_P$ are the constituent mobilities of chloride and protein, respectively. Unfortunately, u_{Cl} cannot be determined directly. This mobility will be lower than that of chloride ions in 0.15 molar sodium chloride because of the viscosity effect of the albumin. However, it would not be correct to divide the chloride ion mobility in 0.15 molar sodium chloride by the relative viscosity of the albumin solution since the viscosity effect involved is different from the macroscopic viscosity because of the great different in size between the ions studied. The viscosity involved here, which may be referred to as a microviscosity, would be expected to be smaller than the macroscopic viscosity. Assuming that the microscopic viscosity effect is the same for sodium and chloride ions

$$u_{Cl} = u^s_{Cl}\, u_{Na}/u^s_{Na} \quad (5)$$

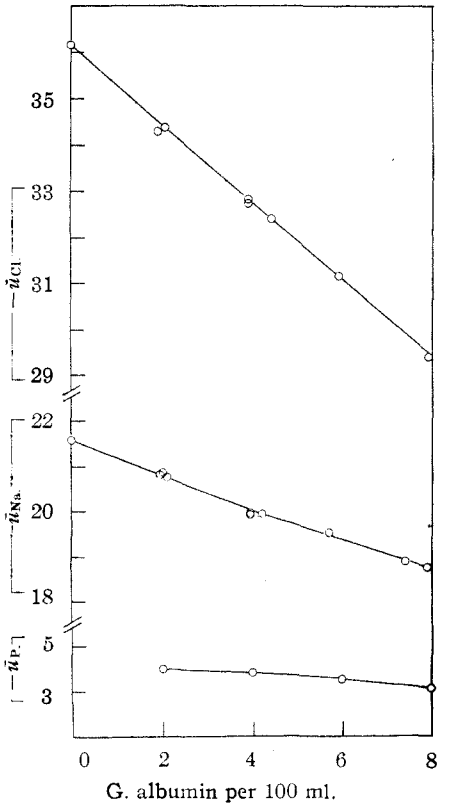

Fig. 2.—Chloride, sodium and protein constituent mobilities in 0.15 molar sodium chloride solutions of albumin at pH 7.0 and 0°.

where the superscript s indicates the mobilities in 0.15 molar salt solution in the absence of albumin. Since not more than a very small amount of sodium ion is bound[5,21] we may assume that the constituent mobility of sodium measured with the system illustrated in Fig. 1(a) is the mobility of *free* sodium ions in the protein solution. Thus equation (4) may be written

$$\bar{v}_{Cl} = \frac{[\bar{Cl}](\bar{u}_{Cl}u^s_{Na} - u^s_{Cl}u_{Na})}{[\bar{P}](\bar{u}_P u^s_{Na} - u^s_{Cl}u_{Na})} \quad (6)$$

In case II the number of equivalents of chloride ion bound per mole of albumin may be calculated from

$$\bar{v}_{Cl} = \frac{[\bar{Cl}](\bar{u}_{Cl} - u_{Cl})}{[\bar{P}](u_P(n-1)/n - u_{Cl} + u_P/n)} + \frac{\bar{u}_P - u_P}{(\bar{u}_P(n-1)/n - u_{Cl} + u_P/n)} \quad (7)$$

where n is the maximum number of chloride ions which may be bound to one albumin molecule and u_P is the mobility of protein with no bound chloride ion. If n is large and $(\bar{u}_P - u_P)$ is small, equation (7) reduces to equation (4).

Case IV: In order to calculate the difference between the number of equivalents of chloride and sodium bound, it may be assumed that the mobility of the protein and the number of ions bound are sufficiently small so that transport of chloride and sodium in protein complexes may be neglected in comparison with that carried by free ions. In this case the constituent mobilities may be written as

$$\bar{u}_{Cl} = \frac{[Cl]}{[\bar{Cl}]}u_{Cl} = \frac{[Cl]}{[\bar{Cl}]}ku^s_{Cl} \quad (8)$$

$$\bar{u}_{Na} = \frac{[Na]}{[\bar{Na}]}u_{Na} = \frac{[Na]}{[\bar{Na}]}ku^s_{Na} \quad (9)$$

where the superscript s again indicates the mobility determined in a solution of the salt at the same ionic strength and k is a proportionality factor for the viscous effect. If it is assumed that k is the same for sodium and chloride ions and that the binding is small, the difference between the binding of chloride and sodium ions may be written with the aid of equations (8) and (9).

$$\bar{v}_{Cl} - \bar{v}_{Na} = \frac{[\bar{Cl}]}{[\bar{P}]}\left(1 - \frac{\bar{u}_{Cl}u^s_{Na}}{\bar{u}_{Na}u^s_{Cl}}\right) \quad (10)$$

Calculations

The constituent mobilities of sodium, chloride and albumin measured in solutions containing 0.15 molar sodium chloride at pH 7.00 are shown in Fig. 2. A smooth curve has been drawn through each set of experimental points, and the mobilities used in the calculations were taken from these curves at albumin concentrations of 2, 4, 6 and 8 g./100 ml. The constituent mobility of sodium was also determined at pH 5.40 in 4% albumin, but it was not possible to obtain a satisfactory moving boundary at pH 3.20. The constituent mobility of chloride was also determined at pH 5.40 and 3.20. The data and the number of equivalents of chloride bound calculated from equations

(21) G. Scatchard, I. H. Scheinberg and S. H. Armstrong, Jr., THIS JOURNAL, **72**, 540 (1950).

TABLE I

CALCULATION OF THE BINDING OF CHLORIDE BY BOVINE SERUM ALBUMIN IN 0.15 *M* SODIUM CHLORIDE AT 0°

*p*H				7.00		5.40	3.20
G. albumin per 100 ml.	0	2	4	6	8	4	4
$\bar{u}_{Cl} \times 10^5$	−36.19	−34.40	−32.72	−31.10	−29.40	−32.62	−29.59
$\bar{u}_{Na} \times 10^5$	21.59	20.84	20.11	19.38	18.65	20.10	20.10[a]
$\bar{u}_P \times 10^5$	...	−4.55	−4.41	−4.26	−4.11	−2.75	4.00[b]
$\bar{\nu}_{Cl}$(eq. 6)		6.3	7.4	7.7	8.2	8.8	29
$\bar{\nu}_{Cl} - \bar{\nu}_{Na}$(eq. 10)		5.5	6.5	6.6	7.1	8.4	32
$\bar{\nu}_{Cl}$(other methods[c])				8		11	31

[a] Assumed. [b] R. A. Alberty, *J. Phys. Colloid Chem.*, **53**, 114 (1949). [c] G. Scatchard, I. H. Scheinberg and S. H. Armstrong, Jr., THIS JOURNAL, **72**, 540 (1950).

(6) and (10) are given in Table I. The experimental uncertainty in $\bar{\nu}$ is of the order of 10% so that the variation in binding with protein concentration at *p*H 7 is not to be considered significant. For comparison, the values calculated from the graph given by Scatchard, Scheinberg and Armstrong for human serum albumin (ref. 5, p. 539) are given in the last line, and it is seen that the agreement is completely satisfactory. The constants in their equation were determined by the dialysis method and the e.m.f. method and allowance was made for two classes of binding sites assuming that the ratio of intrinsic constants is the same as that determined for thiocyanate ion.[21] It is especially interesting that the large increase in binding of chloride at low *p*H which was originally suggested by the titration data of Tanford[22] is confirmed by the moving boundary method. Calculations of binding with equation (7) by making reasonable assumptions as to u_P and n show that this equation yields very nearly the same results as equation (6).

(22) C. Tanford, THIS JOURNAL, **72**, 441 (1950).

Discussion

The moving boundary method for the study of protein–ion interaction has the disadvantage that rather large quantities of the protein are required and very careful mobility determinations must be made. One characteristic of the calculations, however, is that all apparatus factors cancel so that if the same limb of the same Tiselius cell is used for all experiments some possible errors cancel. An advantage of the moving boundary method is that it may be used to study the interactions of proteins with other proteins, nucleic acids or polysaccharides, cases in which the dialyses and e.m.f. methods are not applicable.

Acknowledgments.—The authors are indebted to Dr. Harry Svensson for originally suggesting that it is the constituent mobility which is obtained in experiments of this type. This work was supported by the Research Committee of the Graduate School of the University of Wisconsin from funds supplied by the Wisconsin Alumni Research Foundation.

MADISON, WIS. RECEIVED JANUARY 23, 1951

[CONTRIBUTION NO. 1511 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

The Enzyme–Inhibitor Dissociation Constants of α-Chymotrypsin and Three Enantiomorphic Pairs of Competitive Inhibitors[1]

BY H. T. HUANG AND CARL NIEMANN[2]

The enzyme–inhibitor dissociation constants of α-chymotrypsin and three enantiomorphic pairs of competitive inhibitors derived from tryptophan have been evaluated, at 25° and *p*H 7.9, and it has been found that for each pair of inhibitors the affinity of the enzyme for the D-isomer is greater than that for the L-isomer. The significance of this observation is discussed.

The α-chymotrypsin-catalyzed hydrolysis of specific substrates may be formulated in terms of the Michaelis–Menten intermediate complex theory[3–7] and if consideration is limited to the enzyme and specific substrate[8] the over-all reaction may be visualized as proceeding *via* two consecutive steps, one, the reversible combination of the specific substrate and the enzyme to form a characteristic intermediate enzyme–substrate complex, and two, the subsequent transformation of this complex into enzyme and reaction products. A competitive inhibitor of the above reaction may be regarded as a substance which is capable of participation in only the first step, *i.e.*, reversible combination with the enzyme at the same reactive site involved in the combination of the enzyme with a specific substrate, though it is clear that the distinction between a specific substrate and a competitive inhibitor may

(1) Supported in part by a grant from Eli Lilly and Co.

(2) To whom inquiries regarding this article should be sent.

(3) H. Neurath and G. Schwert, *Chem. Revs.*, **46**, 69 (1950).

(4) H. T. Huang and C. Niemann, THIS JOURNAL, **73**, 1541 (1951).

(5) D. W. Thomas, R. V. MacAllister and C. Niemann, *ibid.*, **73**, 1548 (1951).

(6) R. J. Foster and C. Niemann, *ibid.*, **73**, 1552 (1951).

(7) H. T. Huang and C. Niemann, *ibid.*, **73**, 1555 (1951).

(8) It is obvious that in the hydrolytic reaction the enzyme and specific substrate are not the only reactants since either water or hydroxyl ion must also participate in the reaction. However, as all hydrolytic experiments are usually conducted under conditions wherein these latter "non-specific substrates" are present in great excess little can be said at present in regard to their manner of participation.