tion, which however applies strictly only in the gas phase. Neglecting all internal degrees of freedom of the molecules, the difference between ΔS^0 for the protein Ag–Ab reaction and for the hapten–Ab reaction is written as[28]

$$\Delta(\Delta S^0) = (3R/2) \ln (M_{AgAb} M_{Hp} / M_{HpAb} M_{Ag}) \quad (7)$$

where M_{Ag}, M_{AgAb}, M_{Hp}, M_{HpAb} are the molecular weights of the protein Ag, the Ag–Ab aggregate, the hapten, and the hapten–Ab aggregate, respectively. In the case of BSA-S-R_1, M_{Ag} = 70,000, and for the bivalent hapten M_{Hp} = 564. This gives $\Delta(\Delta S^0)$ of −14 e.u.; *i.e.*, ΔS^0 for the protein Ag–Ab reaction would be 14 e.u. less positive, and ΔF^0 4 kcal./mole more positive, than for the hapten–Ab reaction. The effect is therefore of the right order of magnitude. It should be pointed out that the existence of this effect is due to the particular choice of standard states, in calculations of ΔS^0 and ΔF^0, as solutions containing 1 mole/l. of each of the components of the reaction.

(28) *Cf.*, S. Glasstone, "Textbook of Physical Chemistry," 2nd Ed., D. Van Nostrand Co., New York, N. Y., 1946, p. 874.

Acknowledgments.—We are greatly indebted to Dr. Samuel I. Epstein for having performed preliminary light scattering measurements with the BSA-S-R_1:anti R system and for his advice and guidance with light scattering techniques. We are also grateful to Drs. Alfred M. Holtzer and E. P. Geiduschek for their many helpful suggestions.

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[CONTRIBUTION NO. 1514 FROM THE STERLING CHEMISTRY LABORATORY, YALE UNIVERSITY]

Physical Chemical Studies of Soluble Antigen–Antibody Complexes. XI. An Analysis of the Resolution by Electrophoresis and Ultracentrifugation of a Univalent Antigen–Bivalent Antibody System[1]

BY S. J. SINGER, FRANK A. PEPE AND DAVID ILTEN

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An analysis is presented, with the aid of the Gilbert–Jenkins theory, of the re-equilibration effects attending the resolution by electrophoresis and ultracentrifugation of a univalent antigen (Ag)–bivalent antibody (Ab) system. The calculations show that apparent equilibrium constants calculated from the free Ag area in the ascending electrophoresis patterns and the free Ab area in the descending are smaller than the true values, but by factors not much greater than the experimental error. An independent criterion is evolved for determining the magnitude of the reequilibration effects by a comparison of the free Ag areas in the electrophoresis and ultracentrifuge patterns, which should be widely different if the effects are important.

Introduction

In the accompanying paper[2] an extended physical chemical study is presented of the interaction of a univalent protein antigen (Ag), BSA-S-R_1, with bivalent anti-R antibodies (Ab). This Ag–Ab system is relatively simple in that only two aggregates, AgAb and Ag_2Ab, may form. Estimates of equilibrium constants for the reactions forming these aggregates are obtained from light scattering experiments, which do not disturb the state of equilibrium of the system, and also from an analysis of the schlieren patterns of electrophoresis experiments over a wide range of ratios of BSA-S-R_1 and anti-R Ab. In the electrophoresis experiments, however, partial separation of the components results in a continual disruption of the state of equilibrium, and reactions may be expected to occur throughout the experiment to return the system to that state. These re-equilibration effects may therefore considerably influence the distribution of species in the schlieren patterns. The major purpose of this paper is to assess the significance of the apparent equilibrium constants which we have obtained from the electrophoresis experiments on the BSA-S-R_1:anti-R system, by investigating these re-equilibration effects with the aid of the theory recently developed by Gilbert and Jenkins.[3] Furthermore, the results of this investigation are useful in the interpretation of similar electrophoresis experiments with more complicated protein Ag–Ab systems.[4-7]

The Gilbert–Jenkins theory considers a system of three species in equilibrium, represented by the equation

$$A + B \rightleftharpoons C \quad (1)$$

Now the BSA-S-R_1:anti-R system is more complicated than this: it may be denoted by the simultaneous equilibria

$$\begin{aligned} &(a)\ A + B \rightleftharpoons C \\ &(b)\ A + C \rightleftharpoons D \end{aligned} \quad (2)$$

where A, B, C and D represent the Ag, Ab, AgAb and Ag_2Ab species, respectively. Our justification for using the theory in this case is that it is the most nearly adequate treatment available and should provide at least reliable estimates of the effects produced by reequilibration reactions, particularly since under most conditions in the BSA-S-R_1:anti-R system, the molar concentration of Ag_2Ab turns out to be less than that of AgAb. Furthermore, the mobilities and sedimentation constants of the two species are fairly similar,[2,5,8] and since the species are therefore not well resolved from one another, they may effectively be treated as one, to a first approximation. We shall therefore ignore the effects of reaction 2b in the calculations, and consider them qualitatively subsequently.

(1) This research was supported by grant E-1204C from the National Institutes of Health, United States Public Health Service, and by a grant from the Rockefeller Foundation.

(2) F. A. Pepe and S. J. Singer, THIS JOURNAL, **81**, 3878 (1959).

(3) G. A. Gilbert and R. C. L. Jenkins, *Nature*, **177**, 853 (1956).

(4) S. J. Singer and D. H. Campbell, THIS JOURNAL, **75**, 5577 (1953).

(5) S. J. Singer and D. H. Campbell, *ibid.*, **77**, 3499 (1955).

(6) S. J. Singer and D. H. Campbell, *ibid.*, **77**, 4851 (1955).

(7) M. C. Baker, D. H. Campbell, S. I. Epstein and S. J. Singer, *ibid.*, **78**, 312 (1956).

(8) S. J. Singer and D. H. Campbell, *ibid.*, **74**, 1794 (1952).

Should a more complicated theory become available, a more satisfactory treatment of the data in the accompanying paper[2] can be presented. However, the Gilbert–Jenkins theory serves as a valuable guide in the interpretation of our experimental results, if due regard is paid to the limits of its applicability to our system.

The Gilbert–Jenkins Theory.[3]—In this theoretical treatment of the effect of continuous partial resolution on a system represented by equation 1, it is assumed that the rates of the forward and backward reactions are essentially instantaneous. The effects of diffusion are neglected, and the effects of conductivity and *p*H changes which arise across boundaries during electrophoresis,[9] and the Johnston–Ogston effect[10] in ultracentrifugation are ignored. From this model, the following equations for the molar concentrations of A, B and C, at any position x in the cell after time t, are obtained, starting from an initially sharp boundary at $x = 0$, $t = 0$.

$$a = \frac{k}{\lambda - 1}\left\{1 - \frac{\Phi - \varphi}{\sinh \varphi}\right\} \cosh^2 \frac{\varphi}{2} \qquad (3)$$

$$b = \frac{k}{\lambda + 1}\left\{1 + \frac{\Phi - \varphi}{\sinh \varphi}\right\} \sinh^2 \frac{\varphi}{2} \qquad (4)$$

$$c = ab/k \qquad (5)$$

where φ is a parameter defined by

$$\cosh \varphi = \frac{2(V_C - V_A)(V_C - V_B)}{(V_A - V_B)(V_C - x/t)} - \lambda \qquad (6)$$

$$\lambda = \frac{2V_C - (V_A + V_B)}{V_A - V_B} \qquad (7)$$

In these equations, V_A, V_B and V_C are the velocities of A, B and C, respectively, in the x-direction; k is the reciprocal of the equilibrium constant for the reaction 1; Φ is a constant determined by a_0, b_0 and c_0, the original equilibrium concentrations of A, B and C, respectively, as

$$\Phi = \varphi_0 + \left[\frac{k - a_0(\lambda - 1) + b_0(\lambda + 1)}{k}\right] \tanh \varphi_0 \qquad (8)$$

where φ_0 is determined by

$$\cosh \varphi_0 = \frac{\sqrt{\{a_0(\lambda - 1) + b_0(\lambda + 1) - 2k\}^2 + 8k(\lambda + 1)b_0} + a_0(\lambda - 1) + b_0(\lambda + 1)}{2k} \qquad (9)$$

Experimental and Theoretical Results

Rates of Ag–Ab Reactions.—Before proceeding to the results of the application of this theory to the BSA-S-R_1:anti-R system, we may inquire whether one of the critical assumptions of the theory, that the rates of reactions are essentially instantaneous, is actually met by this system. To determine the order of magnitude of the rates of re-equilibration reactions, the following experiments were performed.[5] A particular solution of BSA-S-R_1 and anti-R was subjected to three different electrophoresis experiments in which the duration of the experiment was varied from 3560 to 21,390 sec., but the total number of coulombs passed in all experiments was the same. Further details of the experimental procedure may be found in the accompanying paper.[2] If the half-times of these reactions were of the order of magnitude of the duration of the experiment, the three patterns should exhibit significant differences. On the contrary, however, the patterns (Fig. 1) are almost identical, any slight differences being attributable to different diffusion times. This is borne out by a detailed area analysis of the patterns. Since other evidence indicates that Ag–Ab reactions are relatively rapid,[8,11,12] it is therefore clear that the half-times of the reactions in this system must be much smaller than 3560 sec. However, whether these rates are effectively instantaneous for the purposes of the Gilbert–Jenkins theory cannot be determined from these experiments.

Theoretical Concentration Distributions in Electrophoresis.—To apply this theory to the electrophoresis experiments described in the accompanying paper, we chose velocities for the different species to correspond closely to the electrophoretic mobilities of BSA-S-R_1 and anti-R Ab at *p*H 8.7, namely, V^E_{Ag}, V^E_{Ab} and V^E_{AgAb} equal to 6, 2 and 4×10^{-5} cm./sec./v./cm., respectively, in both ascending and descending patterns.[13] Three different values of the constant k were introduced, 1×10^{-4}, 1×10^{-5} and 1×10^{-6}, and various values of the original equilibrium molar concentrations of Ag, Ab and AgAb compatible with a particular k value were considered which were similar to those investigated experimentally.[2] The predicted distribution of species for a few of these calculations is indicated in Fig. 2. The molar concentrations of the three species are plotted as functions of the mobility, since the essential shape of this theoretical distribution is independent of time.[9] To compare these theoretical plots with the experimental diagrams of the BSA-S-R_1:anti-R system (Fig. 5 of ref. 2), the former must first be converted to concentration gradient curves and expressed in terms of weight rather than molar concentrations. It should also be noted that because of the neglect of diffusion in the theoretical treatment, some of the concentration gradients are infinite, that is, the boundaries are infinitely sharp.

Several results of this theoretical analysis are particularly interesting. A system in which only reaction 1 occurs, and $V_C^E = (V_A^E + V_B^E)/2$, should exhibit, on a molar basis, certain symmetry properties. Consider two solutions related by the condition $(a_0/b_0)_I = (b_0/a_0)_{II}$. If the electrophoresis patterns were based on a scale of molar concentration, rather than of weight concentration, the descending pattern of I should be identical with the ascending pattern of II, and the ascending of I with the descending of II. A certain degree of symmetry is indeed observed in the electrophoresis of the BSA-S-R_1:anti-R system, if one compares, for example the ascending patterns of Fig. 5 A and B of reference 2, with the descending patterns J and K. The alternative comparison, of the descending patterns of Fig. 5 A and B with the ascending patterns J and K, is not quite as satisfactory, although it should be recognized that these patterns are based on a weight concentration scale and are therefore not strictly comparable in this connection.

The Calculation of Apparent Equilibrium Constants from Electrophoresis Patterns.—An additional prediction of the theory is that the faster boundary in the ascending pattern, and the slower in the descending, should migrate with the mobilities of free Ag and free Ab, respectively. This we have observed experimentally in the BSA-S-R_1:anti-R system.[2] Furthermore, the changes in concentration across these particular two boundaries (the relative "areas" under these peaks on a concentration gradient scale) which are predicted by the theory are both somewhat *larger* than their respective concentrations in the original equilibrium mixture. To understand this qualitatively, consider the ascending limb of the electrophoresis cell. Since Ag and Ag-Ab migrate faster than Ab, leaving the Ab behind, the aggregate must react to produce more Ab and Ag to recover the equilibrium state. This results therefore in the increase in the area under the free Ag peak. A corresponding situation exists in the descending limb, which causes the free Ab peak to increase in area.

In the experimental investigation of the BSA-S-R_1:anti-R system,[2] the observed areas under the free Ag peak in the ascending, and the free Ab peak in the descending patterns, were taken as a first approximation to represent the equilibrium concentrations of free Ag and free Ab, and values of

(9) L. G. Longsworth, *J. Phys. Chem.*, **51**, 171 (1947).

(10) J. P. Johnston and A. G. Ogston, *Trans. Faraday Soc.*, **42**, 789 (1946).

(11) R. J. Goldberg and D. H. Campbell, *J. Immunol.*, **66**, 79 (1951).

(12) B. Gitlin and H. Edelhoch, *ibid.*, **66**, 67 (1951).

(13) The superscript E distinguishes the velocities applying in electrophoresis from those in ultracentrifugation.

apparent intrinsic equilibrium constants, K_i, were calculated from these data. It is clear that these values of K_i are too small, since the true concentrations of free Ag and Ab are overestimated by this procedure. In order to obtain an estimate of the difference between such apparent Ki values and the true ones, the following procedure was applied to the theoretical distributions such as those of Fig. 2. The calculated "areas" under the free Ag and free Ak peaks in the ascending and descending limbs were used to determine apparent Ki values to compare with the originally assumed true value, $K_t = 1/k$. These calculated apparent K_i values are summarized in Table I, for a number of initial ratios of free Ag to free Ab, and for several values of K_t. It is seen that these apparent K_i values are between $^1/_2$ and $^1/_5$ the true values in the range $K_t = 10^4$ to 10^6; the larger the value of K_t, the smaller K_i/K_t.

The K_i values calculated from the apparent free Ag area increase slightly with an increase in the Ag/Ab ratio in the solution, while those calculated from the apparent free Ab area decrease slightly. In the experimental BSA-S-R_1: anti-R system, on the contrary, both sets of K_i values decreased considerably with an increase in Ag/Ab ratio. We therefore considered this trend significant and suggested possible explanations for it in the accompanying paper.[2]

The Reaction Boundary in Electrophoresis.—Besides the free Ag boundary in the ascending patterns and the free Ab boundary in the descending, the theory predicts that another, more complicated, moving boundary should be present in each limb. Across this boundary all three species Ag, Ab and AgAb simultaneously undergo changes in concentration (Fig. 2). Longsworth has suggested[14] that in general this type of boundary be termed a "reaction boundary." It would take us too far afield, in view of the limited objectives of this paper, to give in detail the results of our calculations of the properties of the reaction boundaries in this system. It is of interest, however, to discuss briefly two important features of these boundaries, namely, their mobility and breadth, as derived from these calculations.

TABLE I

CALCULATED APPARENT EQUILIBRIUM CONSTANTS FROM ELECTROPHORESIS

$[Ag]_0$[a] × 10^5	$[Ab]_0$[a] × 10^5	$[AgAb]_0$[a] × 10^5	K_i Ascend.	K_i Descend.
		$K_t \times 10^4$		
2	10	2	0.35 × 10^4	0.48 × 10^4
3	6.67	2	.40	.46
4	5	2	.42	.44
5	4	2	.44	.42
6.67	3	2	.46	.40
10	2	2	.48	.35
		$K_t = 10^5$		
0.74	3.68	2.7	0.22 × 10^5[b]	0.43 × 10^5
1.04	2.60	2.7	.28	.41
1.65	1.65	2.7	.35	.35
2.60	1.04	2.7	.41	.28
3.68	0.74	2.7	.43	.22
		$K_t = 1 \times 10^6$		
0.27	1.34	3.6	0.17 × 10^6	0.42 × 10^6
.38	0.95	3.6	.21	.38
.60	.60	3.6	.29	.29
.95	.38	3.6	.38	.21
1.34	.27	3.6	.42	.17

[a] $[Ag]_0$, $[Ab]_0$, $[AgAb]_0$ are the molar concentrations of free Ag, free b and of aggregate initially present in equilibrium in a solution.

The value of the average mobility of the reaction boundary is between V^E_{Ag} and V^E_{AgAb} in the descending limb, and between V^E_{Ab} and V^E_{AgAb} in the ascending. This mobility increases in both the ascending and descending limbs, as the ratio of Ag to Ab in the original solution increases. The breadth of the reaction boundary, which may be conveniently defined in this context as the range of mobility across which the concentrations of the three species simultaneously undergo change, is always less than $V^E_{AbAg} - V^E_{Ab}$ in the ascending, and $V^E_{Ag} - V^E_{AbAg}$ in the descending limb. The breadth increases markedly with an increase in the association constant $1/k$ in the range 1×10^4 to 1×10^6. At a given value of $1/k$, the breadth of the descending reaction boundary increases, and of the ascending decreases, as the ratio of Ag to Ab in the original solution increases.

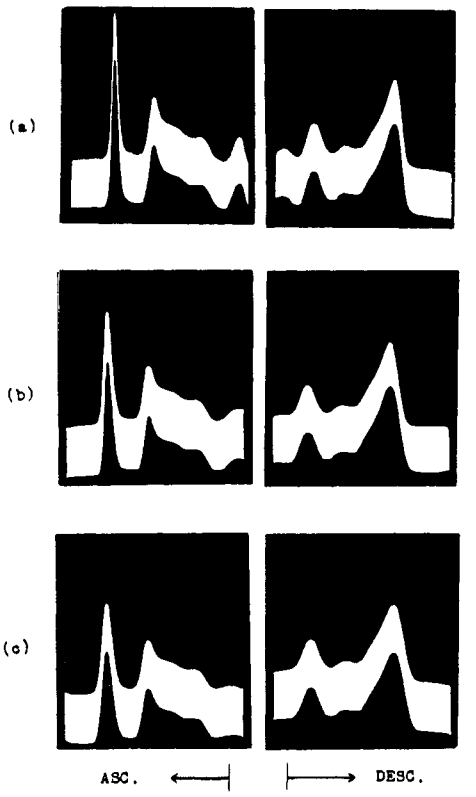

Fig. 1.—Electrophoresis patterns of the same BSA-S-R_1: anti-R mixture at a weight ratio of 0.89 in barbital buffer, pH 8.67, $\Gamma/2$ 0.1 after: (a) 3560 sec. at 0.0120 amp.; (b) 10,700 sec. at 0.0040 amp.; (c) 21,390 sec. at 0.0020 amp. Starting positions and directions of migration are indicated by arrows.

Of special concern for our purposes are the reaction boundaries in the case that $1/k = 1 \times 10^4$, which is the value most nearly appropriate to the BSA-S-R_1:anti-R system.[2] The calculated breadths of these boundaries (Fig. 2) are relatively small, no greater than about 0.3 mobility unit at extreme Ag/Ab ratios. Only for $1/k = 1 \times 10^6$, does the breadth attain values in excess of 1.0 mobility unit. This is in contrast to the experimental behavior of the BSA-S-R_1: anti-R system (Fig. 5 and Table II, ref. 2), for which the reaction boundaries, generally exhibiting two or more maxima in each pattern, are spread over a range of 2.5 to 3.0 mobility units. The neglect of diffusion in the theoretical treatment cannot alone account for this discrepancy, which may be in part the result of reactions involving the $(Ag)_2Ab$ aggregate (equation 2b); or the reaction rates in this system may indeed not be effectively instantaneous as required by the theory.

Re-equilibration Effects in Ultracentrifugation.—Up to this point, our attention has been focused on electrophoretic studies. A few ultracentrifuge experiments were also carried out with the BSA-S-R_1:anti-R system,[3] and it is therefore of interest to use the Gilbert–Jenkins theory to determine the behavior of the corresponding model system during ultracentrifugal resolution. The same equations 3–9 previously used apply, the only differences being the values of V_{Ag}, V_{Ab} and V_{AgAb}, now denoted by V^S, and taken to be equal to 4, 7 and 9 svedbergs, respectively, to correspond to our experimental system.[15]

(14) Personal communication.

(15) F. A. Pepe and S. J. Singer, THIS JOURNAL, **78**, 4583 (1956).

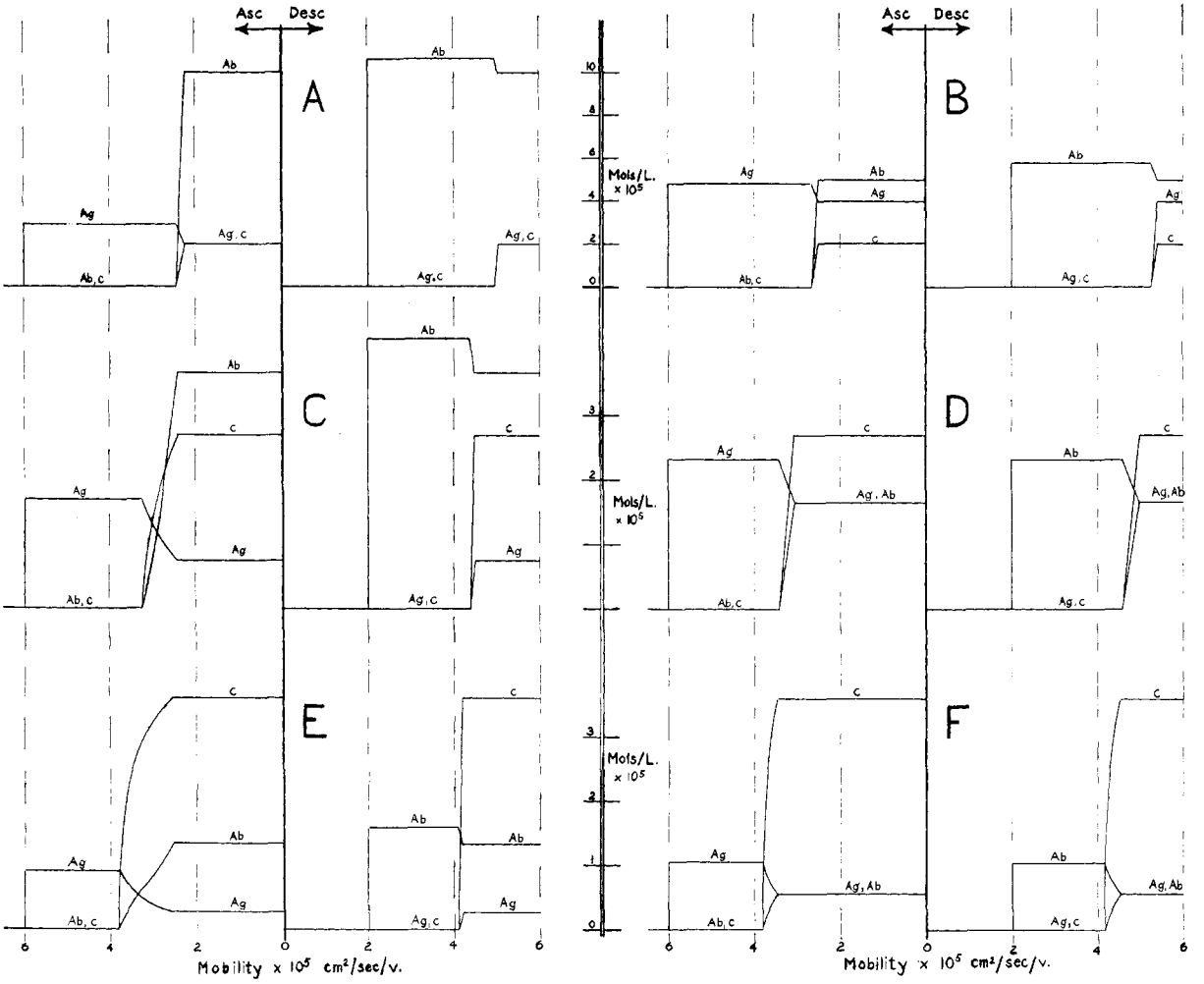

Fig. 2.—Theoretical concentration distributions in ascending and descending electrophoresis patterns of the system Ag + Ab $\rightleftarrows$ c described in the text. In each pair of diagrams, the following values of $[Ag]_0$, $[Ab]_0$, c_0 and k, all $\times 10^5$, apply respectively: (A) 2, 10, 2, 0.1; (B) 4, 5, 2, 0.1; (C) 0.74, 3.68, 2.7, 1.0; (D) 1.65, 1.65, 2.7, 1.0; (E) 0.27, 1.34, 3.6, 10; (F) 0.60, 0.60, 3.6, 10. See also Table I.

The differences in the theoretical re-equilibration behavior of the model system during electrophoresis as compared to ultracentrifugation turn out to be due primarily to the condition that in the former case $V^E_{Ag} > V^E_{AgAb} > V^E_{Ab}$, whereas in the latter, $V^S_{AgAb} > V^S_{Ab} > V^S_{Ag}$. Since the AgAb aggregate is the most rapidly sedimenting species, it leaves behind a region in which the free Ag and free Ab react to produce more aggregate. This has the important consequence that the free Ag "area" in the theoretical ultracentrifuge pattern is *less* than that corresponding to the original equilibrium free Ag concentration. The larger the value of $1/k$, the greater the relative diminution in the free Ag area. Examples of the results of these calculations are given in Table II.

This effect provides, therefore, a useful test of the importance of reequilibration reactions in our experimental system. The relative free Ag area in the ultracentrifuge pattern of a particular solution should be less, whereas the relative free Ag area in the ascending electrophoresis pattern should be greater, than the equilibrium value. These calculations show that the disparity between the ultracentrifugal and electrophoretic results on the same solution should increase with an increase in $1/k$ and should be particularly prominent at low Ag/Ab ratios, if re-equilibration reactions are significant. On the other hand, this test is limited experimentally by the relative inaccuracy of area measurements of ultracentrifuge patterns, as well as by the various anomalies which affect both electrophoresis and ultracentrifuge area distributions.[9,10]

In the BSA-S-R_1:anti-R system, both ultracentrifuge and electrophoresis experiments were performed with three solutions of different Ag/Ab ratios (Fig. 4 of ref. 2). For one of these solutions, however, the degree of Ag excess was too large to permit sufficiently accurate relative free Ag area measurements from the ultracentrifuge patterns. From the ultracentrifuge patterns for the other two solutions, the apparent free Ag concentrations were found to be 14 and 36% of the total protein, whereas the corresponding ascending electrophoresis values, corrected to the same total protein concentration as the ultracentrifuge experiments, were 14 and 33%, respectively. This similarity of the two values for each solution indicates that the effects of re-equilibration reactions are small in this system, which is probably primarily due to the relatively low value of $1/k$ which applies.

Discussion

One of our primary objectives in this paper is to determine the significance of the intrinsic equilibrium constants, K_i, calculated[2] for the BSA-S-R_1:anti-R system from the apparent free Ag and free Ab areas in the ascending and descending electrophoresis patterns, respectively. This problem has been examined by two means. First, insofar as the Gilbert–Jenkins theoretical model applies to the BSA-S-R_1:anti-R system, the calculations of Table

TABLE II

CALCULATED APPARENT EQUILIBRIUM CONSTANTS FROM SEDIMENTATION

$[Ag]_0$[a]	$[Ag]_{app.}$[b]	$[Ab]_0$[a]	$[AgAb]_0$[a]	K_i
		$K_t = 10^4$		
2	1	10	2	3.2×10^4
5	4.3	4	2	1.9
10	9.5	2	2	1.7
		$K_t = 10^5$		
1.04	0.41	2.60	2.7	4.1×10^5
1.65	1.18	1.65	2.7	2.3
2.60	2.27	1.04	2.7	1.9
		$K_t = 10^6$		
0.38	0.068	0.95	3.6	9.0×10^6
0.60	0.38	0.60	3.6	2.6
0.95	0.80	0.38	3.6	2.0

[a] $[Ag]_0$, $[Ab]_0$, $[AgAb]_0$ are the molar concentrations $\times 10^5$ of free Ag, free Ab and of aggregate initially present in equilibrium in a solution. [b] $[Ag]_{app}$ is the apparent molar concentration $\times 10^5$ of free Ag which would be observed in the ultracentrifuge pattern.

I indicate that the average value of $K_i = 1.0 \pm 0.5 \times 10^4$ obtained in the accompanying paper[2] is, if anything, too small, but by no more than a factor of about $^1/_3$. At the present time, such a factor is not much greater than the experimental error. Second, the calculations also reveal that re-equilibration effects should produce opposite results in the ascending electrophoresis compared to the ultracentrifuge patterns: the free Ag area in the former should be greater than in the latter, with the true equilibrium value in between. No significant differences were found in the free Ag areas obtained by the two methods in the experimental system. We conclude, therefore, that the apparent K_i values calculated from the electrophoresis data in the accompanying paper are close to the true values. This conclusion was reached independently[2] from the close correspondence of the equilibrium constants calculated from electrophoresis and light scattering experiments.

That a difference is expected between the free Ag areas in the electrophoresis and ultracentrifuge patterns is a result that is generally useful. It is valid independent of the Gilbert–Jenkins model and applies to multivalent Ag–Ab systems as well as to the simpler BSA-S-R_1:anti-R case. It is the result of the fact that Ag–Ab aggregates, whatever their composition or distribution, always have an electrophoretic mobility between that of free Ag and free Ab,[16] and always have a sedimentation constant greater than that of free Ag and free Ab. If re-equilibration occurs to any significant extent in the ascending electrophoresis limb[16] the aggregates must always react to produce more than the original equilibrium amount of free Ag, whereas in the ultracentrifuge, the free Ag must always be diminished below the equilibrium amount by reactions producing aggregates.

With two multivalent Ag–Ab systems, BSA:anti-BSA[5] and RBSA:anti-R,[7] electrophoretic apparent free Ag areas were found to be not greater than ultracentrifuge values. In fact, in the former system, the latter were slightly larger, probably due to the Johnston–Ogston anomaly. We may conclude therefore that in these systems as well, the apparent K_i values are close to the true ones.

Acknowledgment.—We are grateful to Dr. R. C. Ll. Jenkins for helpful correspondence and advice.

(16) If $|V^E_{Ag}| < |V^E_{Ab}|$, the roles of the ascending and descending limbs are reversed as compared to the BSA-S-R_1:anti-R system, where $|V^E_{Ag}| > |V^E_{Ab}|$.

NEW HAVEN, CONNECTICUT

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, CORNELL UNIVERSITY]

Thermodynamics of the Ionization of the Lysyl Residue of Insulin[1,2]

BY LISE GRUEN,[3] MICHAEL LASKOWSKI, JR., AND HAROLD A. SCHERAGA

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The thermodynamics of the ionization of the ϵ-amino group of the single lysyl residue of insulin have been studied in order to determine whether or not this group is interacting with some other group in the protein. In order to separate the region of ionization of the lysyl residue from that of the four tyrosyl residues, the insulin has been iodinated. All of the tyrosyl residues have been converted to diiodotyrosyls, with no other apparent modification of the protein. By means of titrations in the alkaline pH range at four temperatures and by the direct measurement of the change of pH with temperature, the enthalpy of ionization of the lysyl ϵ-amino group of iodinated insulin has been found to be approximately 13 kcal./mole. For purposes of comparison, the thermodynamics of ionization of the amino groups of n-butylamine, lysine and alanyllysylalanine have been studied by the same methods. The enthalpy of ionization of the ϵ-amino group in the protein agrees well with the values for the model compounds. The apparent pK's and entropies of ionization also agree as well as can be expected, in view of the uncertainties in the electrostatic corrections. If the lysyl group in the protein were involved as the donor in a strong hydrogen bond with some other group, increases of approximately 5 kcal./mole in the enthalpy and 13 e.u. in the entropy over the values observed in model compounds would be expected. These increases are not at all in evidence, indicating either that no lysyl hydrogen bond is present in iodinated insulin or that the bond is too weak to be detected by the methods employed.

Introduction

The location of intramolecular interactions between specific side-chain groups of a protein molecule could be of value in elucidating the spatial configuration of the protein in solution. Insulin is a good subject for this type of study because its amino acid sequence, including the location of the

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(3) National Science Foundation Predoctoral Fellow, 1953–1957.