unsuspected fundamental similarities in the mechanism of reaction between many or all natural protein antigens and their antibodies. In both systems, we have demonstrated[2d,3] that a single ionized carboxyl group is essentially involved in every Ag–Ab bond, and that a positively charged group is presumably present on the complementary site. The positive standard entropy change is attributed to the release of bound, or polarized, water from the charged sites upon bond formation. The near athermicity of these reactions[14] indicates that a fine balance exists among the many factors (including the closeness of approach of oppositely charged groups and of other complementary regions of the two sites; the release of water molecules from these groups and their bonding to other water molecules, etc.) all of which must make substantial contributions to the over-all enthalpy change.[17] It is necessary to examine other natural antigen–antibody systems to determine whether gross Ag variables, such as the degree of antigenicity, molecular weight, shape, electrical properties, etc., have any marked influence on the values of the thermodynamic functions.

(14) A calorimetric value of $\Delta H = -40$ kcal./mole for the reaction between hemocyanin and its antibodies has been reported.[15] This large enthalpy change, however, might be due in part to dissociative equilibria exhibited by hemocyanin,[16] and should be confirmed.

(15) W. C. Boyd, J. B. Conn, D. C. Gregg, G. B. Kistiakowsky and R. M. Roberts, *J. Biol. Chem.*, **139**, 787 (1941).

(16) S. Brohult, *Nova Acta Regiae Soc. Sci. Upsaliensis*, **12**, 1 (1940).

(17) L. Pauling, D. H. Campbell and D. Pressman, *Physiol. Revs.*, **23**, 230 (1943).

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[CONTRIBUTION NO. 1289 FROM THE STERLING CHEMISTRY LABORATORY, YALE UNIVERSITY, AND CONTRIBUTION NO. 1988 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

Physical Chemical Studies of Soluble Antigen–Antibody Complexes. VI. The Effect of pH on the Reaction between Ovalbumin and its Rabbit Antibodies[1]

BY S. J. SINGER, LUTHER EGGMAN AND DAN H. CAMPBELL

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Solutions of soluble complexes formed between ovalbumin (as antigen, Ag) and its rabbit antibodies (Ab) have been subjected to ultracentrifugation over a range of pH. Between pH 4.5 and 3.0 very extensive dissociation of the complexes occurs. Equilibrium constants, K, for the reaction Ag + Ab $\rightleftarrows$ AgAb can be calculated, and the variation of K with pH provides strong evidence that a single ionized carboxyl group is involved in every Ag–Ab bond in this system. The behavior of the rabbit anti-ovalbumin system is remarkably similar to that previously found for the rabbit anti-bovine serum albumin system.

We have previously studied[2] the effect of acid pH on the homogeneous equilibria in the system containing bovine serum albumin (BSA) as antigen (Ag) and its rabbit antibodies (Ab). It was concluded that one ionized carboxyl group is involved in every Ag–Ab bond. A similar investigation has now been made in the system containing ovalbumin (OA) as Ag, and its rabbit antibodies, and the same conclusion has been reached. In fact, the effects of pH on the two systems are quantitatively indistinguishable, further emphasizing the remarkable similarity between the two systems previously observed for the thermodynamic parameters near neutral pH.[3,4]

It has been observed by others[5] that OA–Ab bonds may be dissociated in sufficiently acid or alkaline solutions, and the involvement of oppositely charged groups in the reactive sites has been suggested to account for this. The present more quantitative studies, however, have permitted us not only to eliminate the possibility of other causes for the dissociation, but to define the number of oppositely charged groups critically involved in the Ag–Ab bond.

(1) This work was supported in part by grants from the Rockefeller Foundation and the United States Public Health Service.

(2) S. J. Singer and D. H. Campbell, THIS JOURNAL, **77**, 4851 (1955).

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

(4) S. J. Singer and D. H. Campbell, *ibid.*, **77**, 3504 (1955).

(5) W. J. Kleinschmidt and P. D. Boyer, *J. Immunology*, **79**, 247 (1952).

Materials and Methods

Solution of Ag–Ab Complexes.—In the preceding paper[4] there is described the preparation of a series of solutions (OA-I through OA-I-6) containing only OA and its antibodies. Those portions of OA-I through OA-I-5 which had been subjected to electrophoresis in veronal–NaCl buffer, pH 8.51, $\Gamma/2$ 0.3, were recovered after the experiments, pooled, and pervaporated at 4° to a concentration of 14 mg. protein/ml. (This occurred about one week after the initial preparation of OA-I.) This solution was then dialyzed against phosphate buffer, pH 7.50, $\Gamma/2$ 0.1, and designated OA-R. Aliquots of OA-R to be examined ultracentrifugally were dialyzed 24 hours against buffers of different pH and $\Gamma/2$ 0.1. Electrophoresis in glycine–NaOH buffers, pH 11.70, $\Gamma/2$ 0.1 showed[4] that OA-R contained 62.6% OA and 37.4% Ab.

Ultracentrifugation.—Experiments were carried out with an electrically driven instrument, and area measurements were performed, as previously described.[3,6]

Protein Analyses.—Nesslerization was used for protein analyses, the factor 6.25 being used to convert from mg. N to mg. protein.

pH Measurements.—These were made at 25° on a Beckman Model G instrument.

Experimental Results

Ultracentrifuge experiments were performed with solution OA-R at a protein concentration near 14 mg./ml. in a number of buffers of $\Gamma/2$ 0.1 and pH more acid than 7.5. In acetic buffer, pH 4.98, the ultracentrifuge patterns are very similar to those at pH 7.5. However, below pH 4.5 (in lactic acid–NaOH buffers, $\Gamma/2$ 0.1) profound changes occur. As in the rabbit anti-BSA system,[2] progressively

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

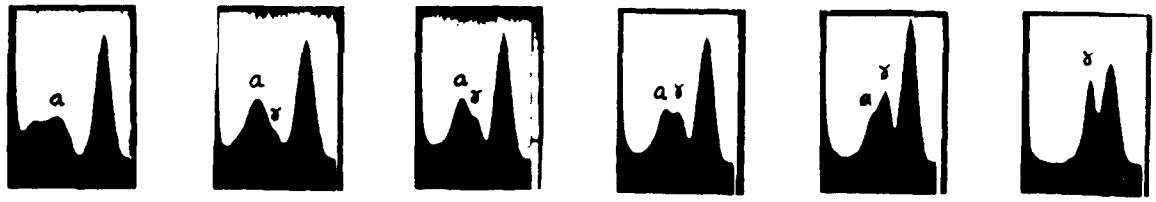

Fig. 1.—The effect of *p*H on the ultracentrifuge diagrams of solution OA-R. Sedimentation proceeds to the left in each diagram. The slowest sedimenting peak is due to free Ag.

larger amounts of free Ab γ-globulin appear between the peaks due to the slowest-sedimenting complex (the **a**-complex) and the free Ag (Fig. 1), while the amounts of the complexes diminish. At *p*H 2.4 (in glycine–HCl buffer, Γ/2 0.1) the diagram is essentially that of a corresponding mixture of OA and normal rabbit γ-globulin. A solution at *p*H 3.1 dialyzed back to *p*H 7.5 exhibits a diagram indistinguishable from that of a solution kept at *p*H 7.5, indicating that the acid dissociation of the complexes was completely reversible under these conditions.

The resolution of the various species, and hence the precision of the relative area determinations of the free Ag, free Ab and **a** complex (Table I, columns 2–4) are poorer than in the rabbit anti-BSA system.[2] Again, we infer from the absence of any other peaks in the diagrams that the AgAb and $(Ag)_2Ab$ complexes are not resolved, and that their sum makes up the **a**-complex area in acid solution. The *fraction* of the **a**-complex area attributable to the AgAb complex (Table I, column 5) is calculated from the relation[2]

$$c_{(Ag)_2Ab} = c^2_{AgAb}/4c_{Ab}$$

TABLE I

EFFECT OF *p*H ON OA–Ab EQUILIBRIA

	Apparent relative areas[b]					
*p*H[a]	Free Ag	Free Ab	a-complex	AgAb[c]	$K^d \times 10^3$	log *K*
4.29	54	6	30	17	14	4.14
4.10	51	9	30	20	12	4.08
3.90	54	15	25	19	6	3.78
3.70	53	17	23	18	5.3	3.72
3.50	54	26	18	16	3.0	3.47
3.30	52	28	14	13	2.4	3.38
3.10	58	32	9	9	1.3	3.11
2.40	60[e]	40	..	..	..	..

[a] Lactic acid–NaOH buffers, Γ/2 0.1, except glycine–HCl buffer, *p*H 2.4, Γ/2 0.1. [b] Given as per cent. of total area expected for total protein concentrations as determined by Nessler analyses. [c] Calculated as described in text. [d] For Ag + Ab ⇄ AgAb, in liter mole^{-1}. [e] 62.6% as determined by electrophoresis[4] in glycine–NaOH, *p*H 11.70, Γ/2 0.1.

where *c* represents molar concentration. This permits evaluation of almost entirely experimental equilibrium constants, *K*, for the reaction Ag + Ab ⇄ AgAb as a function of *p*H[7] (Table I, column 6).

In order to explain the effect of acid *p*H on Ag–Ab equilibria, we have previously[2] proposed a model system with the following properties: (a) in each of the two[4] reactive sites of the Ab molecule there is one negatively charged group, and in all *f* sites of the Ag molecule one positively charged group, which must be ionized for reaction to occur (Interchanging positive and negative charges would have no effect on the argument); (b) the acid dissociation of the positive groups occurs in the alkaline *p*H range; (c) the acid dissociation of the negative groups can be described by a single intrinsic equilibrium constant; (d) the effect on the reaction of the net charges on the entire Ag and Ab molecules is negligible. From this model it follows that

$$\log\left(\frac{1}{K} - \frac{1}{K_O}\right) = \log\frac{K_H}{K_O} - pH \qquad (1)$$

where K_O and K_H are the equilibrium constants for the reactions

$$Ag^{+f} + Ab^{-2} \rightleftharpoons Ag^{+(f-1)}\,Ab^{-1} \qquad (2)$$

$$Ag^{+(f-1)}\,Ab^{-1} + H^+ \rightleftharpoons Ag^{+(f-1)}\,HAb \qquad (3)$$

respectively, and the apparent equilibrium constant, *K*, is determined from the sedimentation patterns.

$$K = \frac{[AgAb]_{total}}{[Ag^{+f}][Ab]_{total}} \qquad (4)$$

If at *p*H ~ 8, the positively and negatively charged groups in the reactive sites of the Ag and Ab molecules are fully ionized, then $K = K_O$ at that *p*H. For the reaction Ag + Ab ⇄ AgAb at *p*H 8.5, *K* was determined[2,9] to be 1.2×10^5. Using this

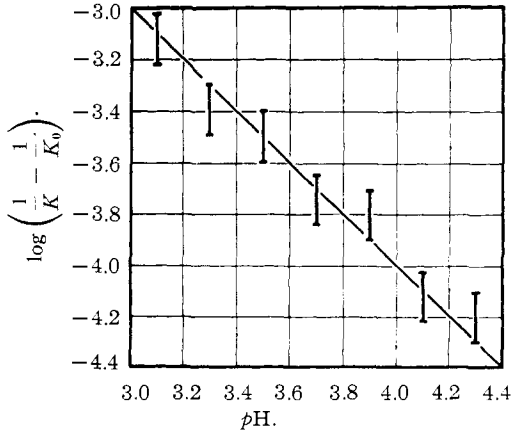

Fig. 2.—The variation with *p*H of equilibrium constants for the reaction Ag + Ab ⇄ AgAb.

(7) No corrections have been made for Johnston–Ogston area anomalies.[2,8] Since the protein concentration was 14 mg./ml. compared to 21 mg./ml. in the anti-BSA study, and the sedimentation constant of OA is smaller than that of BSA, these corrections are less than those previously encountered, and are within experimental error.

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

(9) *K* for Ag + Ab ⇄ AgAb may be taken as 4 × *K* for the reaction Ag + AgAb ⇄ $(Ag)_2Ab$.[10]

(10) R. J. Goldberg, THIS JOURNAL, **74**, 5715 (1952).

value for K_O, and the K values of Table I, we obtain the data plotted in Fig. 2. The relation between log $(1/K = 1/K_O)$ and pH is indeed linear with slope -1.0, within experimental error. Since log $(K_H/K_O) \sim 0$ and $K_H \cong K_O$, the pK for the dissociation of the acid group in the reactive site is about 5, which is near that of an isolated carboxyl group. It is to be noted that the effects of acid pH on the anti-OA and anti-BSA systems are quantitatively indistinguishable.[2]

Our results are therefore consistent with the presence of *one carboxyl* group in each Ag–Ab bond which must be ionized in order for the bond to form. Alternative causes of the acid bond dissociation may be postulated, including (a) non-specific electrostatic repulsion between the positively charged Ag and Ab molecules, and (b) reversible configurational changes in either or both the Ag and Ab molecules. The former of these, however, may be eliminated for the anti-OA system by the same arguments which were valid in the anti-BSA system.[2] As for the latter possibility, while BSA does undergo marked reversible configurational changes in acid solution,[11,12] there is no evidence of similar behavior with OA.[11]

We conclude, therefore, that a single ionized carboxyl group is an essential part of the Ag–Ab bond in the rabbit anti-OA, as well as the rabbit anti-BSA, systems. The inference is made that a single positively charged group is present in each complementary site. which is consistent with (a) the dissociation of OA–Ab bonds in sufficiently alkaline as well as acid solutions[5]; and (b) the positive standard entropy change in the Ag–Ab reaction.[3,4]

Although it has long been suspected that salt linkages constitute a part of the mechanism of the reactions between *natural* protein antigens and their antibodies, quantitative and unambiguous evidence has heretofore been lacking. Whether a single critical salt linkage per bond is a feature common to most, or all, such systems requires further investigation.

(11) C. Tanford, *Proc. Iowa Acad. Sci.*, **59**, 206 (1952).
(12) J. T. Yang and J. F. Foster, THIS JOURNAL, **76**, 1588 (1954).

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[CONTRIBUTION FROM THE DEPARTMENT OF BIOPHYSICAL CHEMISTRY, HARVARD UNIVERSITY]

Interactions of Neutral Amino Acids with Human Serum Albumin and γ-Globulin[1]

BY MARGARET J. HUNTER AND SPENCER L. COMMERFORD

RECEIVED MAY 13, 1955

In order to discover whether or not neutral amino acids were bound by proteins at neutral pH, human serum albumin and human γ-globulin were dialyzed against a tenfold excess of C^{14}-labeled glycine, leucine or phenylalanine, and radioactivity measurements performed on the protein solution and the dialysate. Under the above conditions no binding of the amino acids by the proteins could be demonstrated. An improved technique has been developed whereby protein solutions containing C^{14}-labeled amino acids could be pipetted onto the planchets, dried and counted.

I. Introduction

In the course of studies on the free amino acids of human plasma, it became of interest to determine whether or not these compounds were bound by the proteins of plasma at pH 7.5. In the equilibrium dialysis experiments described below, investigations were restricted to three neutral amino acids: namely, glycine, leucine and phenylalanine, in order to reduce charge effects to a minimum while varying the hydrocarbon side chain. The plasma proteins employed were human serum albumin and human γ-globulin. The amino acids were labeled with C^{14} and the amount of binding was calculated from a comparison of the counts inside and outside the dialysis bag.

II. Materials and Methods

Human Serum Albumin.—The dried powder used as starting material in these experiments had been prepared by fractionation with alcohol and recrystallized three times.[2] An 8% solution of the powder was passed through a mixed-bed de-ionizing resin[3] and the resultant solution frozen at $-5°$ when not in use.

Human γ-Globulin.—The γ-globulins had been prepared by fractionation with alcohol.[4] A 10% solution was twice dialyzed against 10 volumes $\Gamma/2 = 0.1$ NaCl, $\Gamma/2 = 0.05$ phosphate buffer, pH 7.45. Concentrates of the final dialysate were ninhydrin negative. The resultant protein solution was frozen at $-5°$ when not in use.

The C^{14}-labeled amino acids[5] were obtained as solutions of the following molarity and activity:

glycine	0.005 M	2,310,000 c.p.m./ml.
leucine	.025 M	500,000 c.p.m./ml.
phenylalanine	.025 M	100,000 c.p.m./ml.

These were added to the unlabeled amino acid solutions such that the final count was between 2,500 and 100,000 c.p.m./ml. and the molarity of the solution was 10^{-2} or 5×10^{-3}.

Equilibrium Dialysis Procedure.—Two ml. of 10^{-3} M albumin or 5×10^{-4} M γ-globulin was dialyzed in Visking cellulose tubing ($^1/_4$" diam.) against 10 ml. 10^{-2} or 5×10^{-3} M amino acid. In the experiments at low salt concentrations, the amino acids and the protein were brought to pH 7.5 by the addition of 0.1 N NaOH.[6] In the experiments at a higher salt concentration, the ionic strength was 0.15 ($\Gamma/2 = 0.1$ NaCl, $\Gamma/2 = 0.05$ phosphate) and the final pH 7.45. The dialysis was carried out in glass-stoppered tubes at $+1°$ for 24 hours with continuous rotation.

After this time the dialysis bags were removed from the tubes, wiped dry and emptied into small test-tubes. Opti-

(1) This work has been supported by funds of Harvard University, by grants from the National Institutes of Health, and by contributions from industry.

(2) E. J. Cohn, W. L. Hughes, Jr., and J. H. Weare, THIS JOURNAL, **69**, 1753 (1947).

(3) H. M. Dintzis, Dissertation for Ph.D. Thesis, Harvard University, 1953.

(4) J. L. Oncley, M. Melin, D. A. Richert, J. W. Cameron and P. M. Gross, Jr., THIS JOURNAL, **71**, 541 (1949).

(5) The authors are indebted to Dr. R. Loftfield of the Massachusetts General Hospital for the C^{14}-labeled amino acids.

(6) In the experiments with γ-globulin at low ionic strength the 2-ml. aliquots of protein were dialyzed against 500 ml. water, the bags wiped dry and transferred to the dialysis tubes. The final pH of the protein solution in these experiments was always slightly low, about 6.9–7.1.