TABLE I

N-SUBSTITUTED α-PHENYL-α-CARBAMYLACETIC ACIDS AND THEIR METHYL ESTERS $C_6H_5CHCOOH$ (CONRR')

	R	R'	Reagent used	M.p., °C.[b]	Yield,[c] %	Formula	Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found	Neut. equiv. Calcd.	Neut. equiv. Found
1	CH_3	H	CH_3NCO[a]	112–114	29	$C_{10}H_{11}O_3N$	62.16	62.16[d]	5.74	5.83	193.2	193.6
2	Methyl ester of 1			121–123		$C_{11}H_{13}O_3N$	63.75	63.84	6.32	6.32		
3	C_2H_5	H	C_2H_5NCO	110–112	57	$C_{11}H_{13}O_3N$	63.75	63.52	6.32	6.11	207.2	208.0
4	Methyl ester of 3[e]			81–83		$C_{12}H_{15}O_3N$	65.13	65.43	6.85	7.08		
5	C_6H_5	H	C_6H_5NCO	132–133	67	$C_{15}H_{13}O_3N$	70.58	70.83	5.13	5.10	255.3	256.0
6	Methyl ester of 5[f]			111–113[g]								
7	C_2H_5	C_2H_5	$(C_2H_5)_2NCOCl$	91–92	71	$C_{13}H_{17}O_3N$	66.36	66.49	7.28	7.29	235.3	236.1
8	Methyl ester of 7			64–66		$C_{14}H_{19}O_3N$	67.44	67.48	7.68	7.66		
9	C_6H_5	C_6H_5	$(C_6H_5)_2NCOCl$	121–122	47	$C_{21}H_{17}O_3N$	76.12	76.19	5.17	5.26	331.4	331.9
10	Methyl ester of 9			113–115		$C_{22}H_{19}O_3N$	76.50	76.75	5.55	5.68		

[a] Prepared by the method of J. Colucci, *J. Can. Research*, **23B**, 111 (1945). [b] All of the acids melted with the evolution of a gas. [c] Yields based on phenylacetic acid. [d] Because of the instability of this compound, it was necessary to analyze it immediately after isolation. [e] Calcd.: N, 6.33. Found: N, 6.27. [f] Calcd.: N, 5.20. Found: N, 5.16. [g] H. Staudinger and H. Hurzel, *Ber.*, **50**, 1031 (1917), report m.p. 109°.

pyl ether; yield 9.5 g. After further recrystallization from diisopropyl ether, the compound melted at 82–84°, mixed m.p. 82–84°.

Compound I was treated with 0.12 mole of phenyl isothiocyanate in the manner described above; the product was isolated by procedure B. The crude material was triturated with 50 cc. of diisopropyl ether and then refrigerated for 12 hours; yield 13.5 g. The yellow α-phenylthioacetanilide melted at 86.5–87.5°[11]; after recrystallization from diisopropyl ether, mixed m.p. with an authentic sample[11,12] 86.5–87.5°.

(11) F. Sachs and H. Loevy, *Ber.*, **37**, 875 (1904), m. p. 87°.

(12) We obtained the product in 80% yield by the method of Sachs and Loevy.[11]

ANN ARBOR, MICHIGAN

[CONTRIBUTION NO. 1288 FROM THE STERLING CHEMISTRY LABORATORY, YALE UNIVERSITY, AND CONTRIBUTION NO. 1986 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

Physical Chemical Studies of Soluble Antigen–Antibody Complexes. V. Thermodynamics of the Reaction between Ovalbumin and its Rabbit Antibodies[1]

BY S. J. SINGER AND DAN H. CAMPBELL

RECEIVED APRIL 4, 1955

Solutions of soluble complexes formed between ovalbumin (as antigen) and its rabbit antibodies have been subjected to electrophoresis and ultracentrifugation. It has thus been possible to determine the equilibrium concentration of uncombined antigen (Ag) in a given solution of known total antigen and total antibody (Ab) content. With the aid of the Goldberg theory, the data have been interpreted to give the following information for the reaction $Ag + AgAb \rightleftarrows (Ag)_2Ab$ in veronal–NaCl buffer, *p*H 8.5, Γ/2 0.3, at 0°: $K = (3.1 \pm 0.5) \times 10^4$, $\Delta F^\circ = -5.6 \pm 0.2$ kcal./mole, $\Delta H^\circ = 0 \pm 2$ kcal./mole, and $\Delta S^\circ = +20 \pm 8$ e.u. These data are remarkably similar to those previously found for the rabbit antibovine serum albumin system, and their significance is discussed.

In previous papers of this series,[2] ultracentrifugal and electrophoretic studies were described with the soluble complexes formed between bovine serum albumin (BSA) and its rabbit antibodies. A general method was introduced[2b,c] for the evaluation of thermodynamic data for antigen (Ag)–antibody (Ab) reactions, which involves essentially the electrophoretic determination of the amounts of both the total Ag and the free Ag in equilibrium in a solution. The method was applied to the rabbit anti-BSA system,[2c] and independent tests confirmed its validity.[2d] We have now investigated the system containing ovalbumin (OA) and its precipitating rabbit antibodies. The thermodynamic data for these two systems are remarkably similar, and suggest that a basic mechanism, more detailed than previously recognized, may be common to all antigen–antibody reactions. This suggestion is further implemented in the following paper.[3]

Materials and Methods

Ovalbumin.—The protein was a four-times recrystallized preparation[4] which was lyophilized for storage. About 95% of the protein redissolved in neutral buffers to give an ultracentrifugally homogeneous material. Upon centrifuging off the insoluble residue, the solution remained clear for the time necessary to perform the following experiments.

Solutions of Soluble Complexes.—Two preparations, OA-I and OA-II, were made as previously described for solution VI in the rabbit anti-BSA system,[2c] except that the pooled hyperimmune antisera were never frozen. In dissolving the specific Ag–Ab precipitate in excess OA, it was found necessary to use no more than gentle stirring in order to avoid denaturation of OA.

Solutions OA-I-1 through OA-I-6, in increasing OA excess, were prepared by weight by the addition of a standard OA solution to OA-I.[2c] Solution OA-I-4a, used in the re-

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

(2) (a) S. J. Singer and D. H. Campbell, THIS JOURNAL, **74**, 1794 (1952); (b) **75**, 5577 (1953); (c) **77**, 3499 (1955); (d) **77**, 3504 (1955).

(3) S. J. Singer, L. Eggman and D. H. Campbell, *ibid.*, **77**, 4855 (1955).

(4) S. P. L. Sørensen and M. Høyrup, *Compt. rend. trav. lab. Carlsberg*, **12**, 12 (1915/1917).

equilibration studies, was prepared by adding together by weight some of solutions OA-I, OA-I-3 and standard OA; it contained 75% total Ag.

Normal Rabbit γ-Globulin.—The preparation RGG-II[2c] containing 98% γ-globulin, was used to make up carefully prepared mixtures with OA, for the calibration experiments discussed below.

Protein Analyses.—Refractive increments determined with a Brice-Phoenix differential refractometer were employed for protein analyses.[2c]

Ultracentrifugation and Electrophoresis.—The ultracentrifuge experiments on preparation OA-I were performed with an electrically driven instrument previously described[2a]; with OA-II, a Spinco Model E machine was used. Electrophoresis was carried out in a Perkin–Elmer Model 38 Tiselius apparatus. Area measurements were made as before.[2b,c]

***p*H Measurements.**—A Beckman Model G *p*H meter was utilized at 25°. A correction was applied to the *p*H observed with the ordinary glass electrode for the glycine–NaOH buffer.

Results

Effects of Re-equilibration during Electrophoresis.—In solutions of Ag–Ab complexes, a variety of species exist in equilibrium. The effects of re-equilibration during an electrophoresis experiment may complicate the conventional interpretation of the schlieren diagrams obtained. A test of the importance of these effects was developed and was previously applied to the rabbit anti-BSA system.[2c] Several experiments with aliquots of the same Ag–Ab solution in the same buffer are performed, in which the number of coulombs passed through the cell, and hence the distances moved by the various boundaries, are the same, but the current and duration of the experiments are varied inversely. If the apparent area under the leading peak (the free Ag peak) in the ascending patterns is constant, then the effects of re-equilibration are negligible. Experiments with solution OA-I-4a in veronal buffer, *p*H 8.61, Γ/2 0.1 (Fig. 1 and Table I) indicate that in this, as in the rabbit anti-BSA system, these effects may be ignored for our purposes.

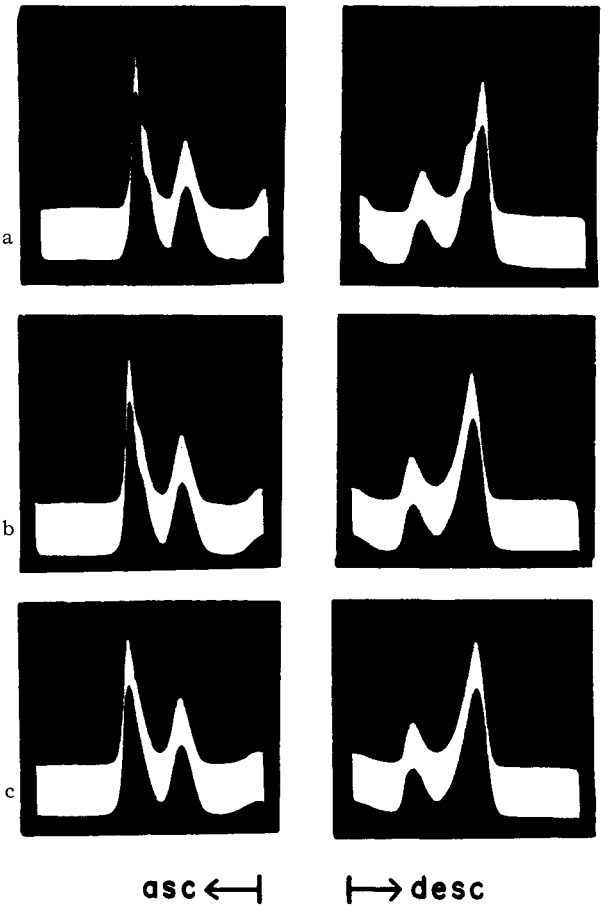

Fig. 1.—Electrophoresis of solution OA-I-4a, in veronal buffer, *p*H 8.61, Γ/2 0.1: (a) 3000 sec., 0.0120 amp.; (b) 6000 sec., 0.0060 amp.; (c) 10,285 sec., 0.0035 amp.

TABLE I

EFFECT OF TIME ON ELECTROPHORESIS PATTERNS[a]

Current, mamp.	Time, sec.	Apparent % free Ag
3.5	10,285	62.6
6.0	6,000	62.5
12.0	3,000	62.7

[a] Experiments in veronal buffer, *p*H 8.60 Γ/2 0.1, see text.

There is a partial splitting of the free Ag peak in the ascending patterns, which is due to the electrophoretic heterogeneity of ovalbumin.[5] In the longer electrophoretic runs, diffusion partially obscures this splitting and causes the leading peak to appear asymmetric (see also Fig. 2).

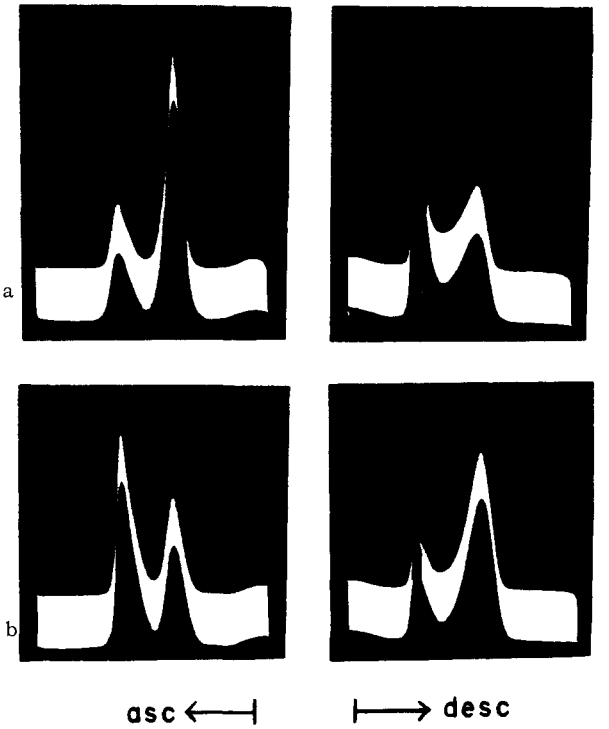

Fig. 2.—Electrophoresis of OA-Ab solutions in veronal–NaCl buffer, *p*H 8.51, Γ/2 0.3: (a) solution OA-I-1; (b) solution OA-I-4.

Electrophoresis Results.—For the determination of the equilibrium amounts of free Ag, solutions OA-I through OA-I-6 were examined electrophoretically at a total protein concentration of 14 mg./ml. in veronal–NaCl buffer, *p*H 8.52, Γ/2 0.3 (Fig. 2). In this buffer, area anomalies are reduced to within experimental error in a system of this type.[2c] All experiments were run for 14,300

(5) L. G. Longsworth, THIS JOURNAL, **61**, 529 (1939).

sec. at a field strength of 4.16 volts/cm. As discussed previously,[2a,c] the equilibrium per cent. of free Ag in a solution is taken as the relative area under the leading ascending peak (Table II, column 2).

TABLE II

ELECTROPHORETIC DATA AND EQUILIBRIUM CONSTANTS

Soln.	% free Ag[a]	% Total Ag Electroph.[b]	% Total Ag Analyt.	$K^c \times 10^{-4}$
OA-I	16.7	38.4	(38.4)	3.2
OA-I-1	25.8	..	45.7	1.8
OA-I-2	32.2	52.3	52.5	3.1
OA-I-3	45.1	61.3	61.8	2.5
OA-I-4	60.6	..	73.4	3.6
OA-I-5	73.1	..	82.1	4.4
OA-I-6	87.8	..	91.3	0.5[d]

[a] The relative area of free Ag peak in ascending pattern, in veronal–NaCl buffer, *p*H 8.51, Γ/2 0.3. [b] The relative area of Ag peak in ascending pattern, in glycine–NaOH buffer, *p*H 11.70, Γ/2 0.1. [c] For the reaction Ag + AgAb $\rightleftarrows$ $(Ag)_2Ab$. [d] Low value due to large uncertainty in small difference between two large numbers. (If % free Ag is 87.0, $K = 3.0 \times 10^4$.) Not included in the average.

The electrophoretic patterns (Figure 2) are similar to those obtained with the rabbit anti-BSA system, two principal peaks, due to free Ag and to complexes, appearing in both ascending and descending limbs. The only significant difference is that the peak due to the complexes (the slower peak) in the descending patterns is partially obscured by a region of pronounced turbidity. Clearly this turbidity is attributable to the fact that the free Ag has migrated away from the complexes, permitting them to adjust to new equilibrium conditions closer to the precipitation zone. This situation does not obtain in the ascending limb.[2a] This indicates, therefore, that the rates of reactions are greater in the OA than in the BSA system.

For the electrophoretic determination of total Ag, conditions must be found in which the Ag–Ab bonds are completely dissociated, and the Ag and Ab γ-globulin which result remain completely soluble and are electrophoretically resolvable. In the rabbit anti–BSA system, such analyses were performed in glycine–HCl buffer, *p*H 2.4, Γ/2 0.1, but the OA and Ab γ-globulin mobilities are too nearly alike at this and more acid *p*H. On the other hand, the alkaline dissociation and electrophoresis of OA anti-OA have previously been reported.[6] Accordingly, solutions OA-I, OA-I-2 and OA-I-3, at total protein concentrations of 14 mg./ml. were examined in glycine–NaOH buffer, *p*H 11.70, Γ/2 0.1 (Fig. 3) and the relative area under the leading ascending peak was taken as the per cent. total Ag (Table II, column 3). In order to eliminate uncertainties due to area anomalies, calibration experiments were performed in the same buffer with carefully prepared mixtures[2c] of OA and normal rabbit γ-globulin (preparation RGG-II). The per cent. OA, obtained by as closely similar resolution of the ascending patterns as was employed for the Ab-

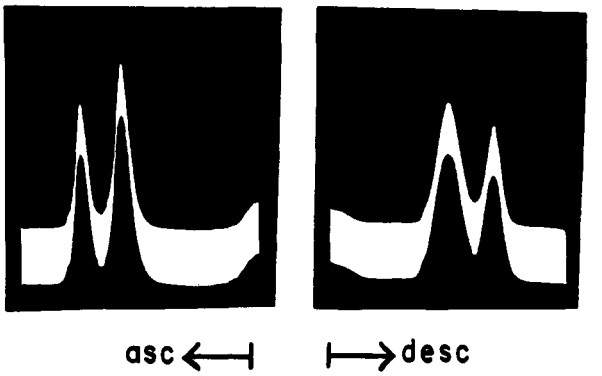

Fig. 3.—Electrophoresis of solution OA-I in glycine–NaOH buffer, *p*H 11.70, Γ/2 0.1.

containing solutions, was within experimental error the correct value (Table III).

TABLE III

ELECTROPHORETIC DETERMINATION OF OA IN MIXTURES WITH NORMAL RABBIT γ-GLOBULIN

Electroph.[a]	41.0	50.4	57.5	68.7
Analyt.	39.9	50.4	58.6	69.5

[a] Relative OA area in ascending pattern, in glycine–NaOH buffer, *p*H 11.70, Γ/2 0.1.

From the per cent. total Ag determined electrophoretically for solution OA-I, and from the proportions in which OA-I and the standard OA solution were mixed, the compositions of OA-I-1 through OA-I-6 were calculated (Table II, column 4). The good agreement between the electrophoretic and analytical values for OA-I-2 and OA-I-3 provides an additional check.

In all our electrophoretic and ultracentrifugal studies of OA anti-OA solutions, there was no indication of the presence of any species other than Ag, Ab and aggregates of the two.

Ultracentrifuge Results.[7]—Four solutions from preparation OA-I were examined ultracentrifugally in phosphate buffer, *p*H 7.50, Γ/2 0.1 (Fig. 4). The principal use of these experiments is qualitative, since the area measurements are less accurate than those from electrophoresis and are further complicated by the Johnston–Ogston anomaly.[2c,8] It is significant that the distribution of species in the sedimentation diagrams, and the variation with the degree of Ag excess, are strikingly similar to the rabbit anti–BSA system (Fig. 2 of reference 2a), taking into account the difference in molecular weight of the two antigens. The nature of the various species is therefore given by analogy,[2a] the slowest-moving complex (the **a** complex) being assigned the structure $(Ag)_2Ab$. The sedimentation patterns are entirely consistent with bivalence of the antibody[2a,c] (see below). Furthermore, the build-up of larger complexes as the zone of precipitation is approached provides additional direct evidence in support of the framework theory of Ag–Ab reactions.[9]

(6) W. J. Kleinschmidt and P. D. Boyer, *J. Immunology*, **69**, 247, 257 (1952). Contrary to their findings, our results (Fig. 3) indicate that OA–Ab complexes are completely dissociated at *p*H 11.7. The discrepancy might be due to different extents of alkaline denaturation of Ab. For analytical purposes, denaturation would not interfere as long as all the protein remained soluble.

(7) We are indebted to Dr. Luther Eggman for performing the experiments on preparation OA-I.

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

(9) J. R. Marrack, "The Chemistry of Antigens and Antibodies," Med. Res. Council, Special Report Series No. 230, London, 1938.

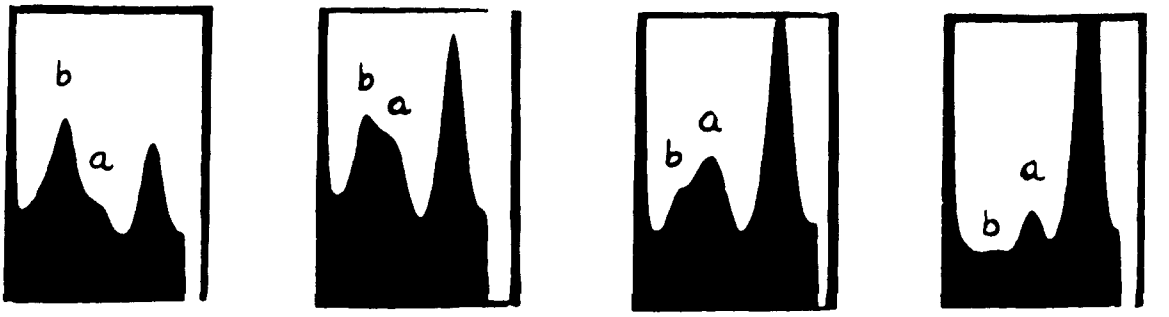

Fig. 4.—Comparable ultracentrifuge diagrams of (reading left to right) solutions OA-I, OA-I-2, OA-I-3 and OA-I-6, after about 5000 sec. at 54000 r.p.m. Sedimentation proceeds to the left in each diagram. The slowest sedimenting peak is the free Ag; other peaks are due to the **a** and **b** complexes.

In order to measure ΔH for the Ag–Ab reaction, ultracentrifuge experiments were performed with OA-II at 3 and 36° (Fig. 5), and careful measurements of the change in the free Ag area were made.[2c] Within a precision of $\pm 2\%$, no such change can be detected. As with the rabbit anti-BSA system,[2c] the region of the patterns between the **a** and b complexes is better resolved at the lower temperature, presumably due to slower re-equilibration.

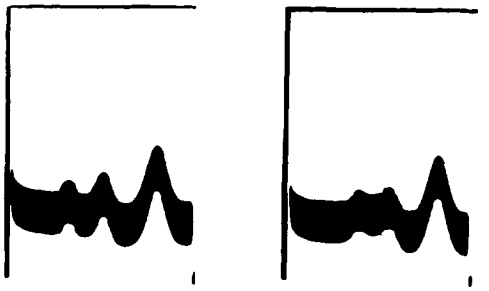

Fig. 5.—Comparable ultracentrifuge diagrams of solution OA-II at two different temperatures.

Discussion

The Valence of Antibody.—We have previously demonstrated[2a,c] that precipitating rabbit antibodies to BSA are largely, if not all, bivalent. The same conclusion holds for the rabbit anti-OA system. The amount of Ag bound in all the complexes in a solution is given by the difference between the total Ag and free Ag. With the approximation that all Ab is bound in complexes, and with molecular weights of 44,000 and 160,000 for Ag and Ab, respectively, we may obtain $\overline{(Ag/Ab)}_{B,N}$, the average number of Ag molecules bound per Ab molecule in all the complexes in a solution.

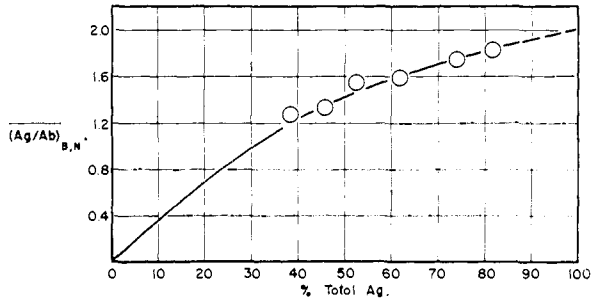

Fig. 6.—The binding of antigen to antibody, see text.

The limit approached by this number in great antigen excess should be the antibody valence, and the data of Table II lead to a valence of 2 (Fig. 6).

Equilibrium Constants and Thermodynamic Parameters.—For the determination of equilibrium constants, K, the free Ag and total Ag concentrations are used in conjunction with part of the Goldberg theory,[10] as completely described elsewhere.[2c] K for the reaction Ag + AgAb $\rightleftarrows$ $(Ag)_2Ab$ may be calculated (Table II, column 5), taking the valence of Ab as 2, and of Ag as 5.[11] (For Ag valence > 4, K is essentially independent of f.) Within experimental error, K does not vary with the degree of Ag excess, and its average value is $(3.1 \pm 0.5) \times 10^4$. From this, the standard free energy change is $-(5.6 \pm 0.2)$ kcal. per mole (for the reaction Ag + AgAb $\rightleftarrows$ $(Ag)_2Ab$ in veronal–NaCl buffer, pH 8.51, $\Gamma/2$ 0.3 at 0°).

Within the precision of the measurements, there is no effect of temperature on the equilibria in this system. From the relation $d \ln K/dT = \Delta H^\circ/RT^2$, one can calculate that an uncertainty of $\pm 2\%$ in detecting a change in the free Ag area in the temperature interval 3–36°, corresponds to an uncertainty of ± 2 kcal. in the enthalpy change for the reaction. Hence, $\Delta H^\circ = 0 \pm 2$ kcal. per mole. These results lead, from the relation $\Delta F^\circ = \Delta H^\circ - T\Delta S^\circ$, to a standard entropy change of $+(20 \pm 8)$ e.u.

The values of K, ΔF° and ΔS° should be considered as effective values averaged over any Ag or Ab heterogeneity which may exist in this system. Ovalbumin is electrophoretically heterogeneous,[5] but this is due to the presence of three fractions containing two, one, and no phosphate groups per molecule, respectively.[12] Presumably, the fractions are otherwise identical, as is suggested by terminal amino acid analysis.[13] Since the phosphate groups do not appear to be involved in the antigenic sites, the electrophoretic heterogeneity does not imply any antigenic heterogeneity.

It is remarkable that the thermodynamic data for the rabbit anti-OA and anti-BSA[2c] systems are the same (within current experimental error), in view of the facts that the two antigens are unrelated serologically, and have quite different molecular weights. These data may reflect hitherto

(10) R. J. Goldberg, THIS JOURNAL, **74**, 5715 (1952).
(11) W. C. Boyd, in H. Neurath and K. Bailey, "The Proteins," Vol. II, Academic Press, Inc., New York, N. Y., 1954, p. 777.
(12) G. E. Perlmann, *J. Gen. Physiol.*, **35**, 711 (1952).
(13) R. R. Porter, *Biochem. J.*, **46**, 473 (1950).

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).

NEW HAVEN, CONN.
PASADENA, CALIFORNIA

[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

RECEIVED APRIL 4, 1955

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).