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Physical Chemical Studies of Soluble Antigen–Antibody Complexes. I. The Valence of Precipitating Rabbit Antibody[1]

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A quantitative ultracentrifugal and electrophoretic study has been performed with the soluble antigen–antibody complexes formed in antigen excess between lightly-iodinated bovine serum albumin antigen and precipitating rabbit anti-bovine serum albumin antibodies. A particular species of complex, the **a** complex, is found to predominate in large antigen excess, and it is demonstrated that this complex must be largely the species containing two antigen molecules and one antibody. It is concluded, therefore, that the antibodies in this system are largely bivalent.

A specific precipitate formed between an antigen and its antibody can be dissolved by adding to it a sufficient excess of the antigen in solution. Heidelberger and Pedersen[3] studied such solutions, containing egg albumin antigen and rabbit antibodies, in the ultracentrifuge. They demonstrated the presence of distinct molecular species in solution, sedimenting more rapidly than either antigen or antibody molecules, and concluded that these species were a number of definite complexes formed between the antigen and antibody. Pappenheimer, Lundgren and Williams[4] subsequently investigated the diphtheria toxin–horse antitoxin system in the ultracentrifuge. Mixtures of the toxin with the antitoxic pseudoglobulin in the region of toxin excess revealed the presence of complexes sedimenting faster than either the toxin or the inert pseudoglobulin in the solution. Ultracentrifuge experiments which we performed[5] with the bovine serum albumin (BSA)–rabbit anti-BSA system in antigen excess also demonstrated the existence of several species of complexes. Becker and Munoz[6] have recently reported similar results for similar systems.

We concluded from our preliminary experiments that an extensive quantitative study of the ultracentrifugal and electrophoretic behavior of these systems of soluble antigen–antibody complexes could yield valuable information about the nature of the immunologic reaction. In particular, knowledge about the structure of antibodies and of the free energy of formation of the bond between antigen and antibody might be forthcoming from such a study. We accordingly carried out a quantitative investigation of the rabbit anti-BSA system, the results of which are reported in this paper. These results have led to the conclusion that the precipitating antibodies in this system are largely bivalent. The problem of antibody valence is still the subject of controversy and in the last few years evidence of apparently conflicting nature has been obtained by a variety of methods.[7] Most of the differences center about the question as to whether precipitating antibodies are largely univalent or bivalent; possible higher valences are usually not considered, at least not for rabbit antibodies.

Experimental Methods

Preparation of Solutions of Antigen–Antibody Complexes.—Our preliminary experiments[5] utilized BSA and rabbit antibodies to BSA. In the course of these experiments a method was developed for preparing solutions of soluble antigen–antibody complexes in a region of antigen excess much closer to the equivalence zone than was possible by other means, and this method has been used in the studies reported in this paper. This method, however, renders it impossible to determine the exact amounts of total antigen and total antibody in the final solutions unless one of the two proteins is labeled. Since lightly labeling the antigen might be expected to have the lesser effect on the antigen–antibody reaction, and since iodination is a simple and well-studied method of labeling proteins, the possible use of iodinated bovine serum albumin (BSAI) in this research was investigated.

The effect was determined of different degrees of iodination of BSA upon the precipitin test between BSAI and antisera to un-iodinated BSA. Eisen and Keston[8] have investigated this problem for the case that on the average only one BSA molecule in five or ten is iodinated, using I^{131}, and they found no effect of this degree of iodination on the precipitin reaction. For our purposes we required higher degrees of iodination than were examined by these authors. Four preparations, containing an average of 2.4, 5.9, 13.1 and 20.8 I atoms per BSA molecule, were made. A γ-globulin fraction from pooled high titer anti-BSA sera was prepared by precipitation with $^1/_3$ saturated $(NH_4)_2SO_4$, and subsequent solution of the precipitate in saline at *p*H 7.5. The results of the precipitin tests with this antibody preparation and BSAI and BSA antigens are given in Table I. No significant effect on the precipitin test was produced by the iodination of the BSA to the extent studied.

TABLE I

BSAI–ANTI-BSA PRECIPITIN TESTS

Antigen added, mg.	Total protein precipitated[a] BSA	2.4 I	5.9 I	13.1 I	20.8 I
0.0156	0.83	0.86	0.89	0.30	0.90
.0313	1.7	1.4	1.7	1.4	1.5
.0625	2.5	2.9	2.6	2.7	2.4
.125	2.4	2.7	2.8	2.8	2.9
.250	0.81	1.0	0.85	0.96	1.1

[a] Mg. per ml. of original antibody-containing γ-globulin solution.

Further tests of possible alterations in the immunologic reaction produced by iodinating the BSA will be described

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(2) Chemistry Department, Yale University.

(3) M. Heidelberger and K. O. Pedersen, *J. Exptl. Med.*, **65**, 393 (1937).

(4) A. M. Pappenheimer, Jr., H. P. Lundgren and J. W. Williams, *ibid.*, **71**, 247 (1940).

(5) Unpublished experiments.

(6) E. L. Becker and J. Munoz, paper presented before the Meeting of the American Chemical Society, Chicago, Ill., September, 1950.

(7) L. Pauling, D. Pressman and D. H. Campbell, THIS JOURNAL, **66**, 330 (1944); H. N. Eisen and F. Karush, *ibid.*, **71**, 363 (1949); L. Lerman, Ph.D. Thesis, California Institute of Technology, 1950; T. E. Banks, G. E. Francis, W. Mulligan and A. Wormall, *Biochem. J.*, **48**, 371 (1951); F. Haurowitz, "Chemistry and Biology of Proteins," Academic Press, Inc., New York, N. Y., 1950, p. 290; J. R. Marrack, H. Hoch and R. G. S. Johns, Abstr. of Proc. Biochem. Society, *Biochem. J.*, **48**, xxi (1951).

(8) H. N. Eisen and A. S. Keston, *J. Immunol.*, **63**, 71 (1949).

TABLE II

ULTRACENTRIFUGE RESULTS

Soln. no.	Total protein, mg./ml.	$(AG/AB)_w$	BSA-5I	s^{w}_{20} a complex	b complex	BSA-5I	Relative areas a complex	b complex	h complex	a/Σ[a]	a/b
I	18.0	0.53	3.9	..	11.2	..	5	23	..	..	0.22
I-a	12.0	.53	...	..	10.9	..	..	17	47	..	..
I-b	11.1	.53	...	..	11.3	..	..	23	..	..	..
I-c	6.6	.53	...	..	12.0	..	..	20	..	..	..
II	18.0	.56	3.8	8.06	11.0	16	7	20	..	8	0.35
II-a	12.0	.56	4.0	8.31	11.2	15	5	15	..	6	0.33
II-b	8.0	.56	4.1	8.22	11.3	15	5	16	..	6	0.31
II-c	5.3	.56	4.2	..	11.7	20	7	..	..	8	..
I-1	15.7	1.00	4.1	8.31	11.0	30	10	..	..	14	..
I-1-a	10.4	1.00	4.0	..	11.1	30	10	..	..	14	..
I-1-b	7.0	1.00	4.3	..	12.0	33	10	..	..	15	..
I-1-c	4.6	1.00	4.1	8.27	11.7	33	9	..	..	14	..
II-1	16.3	1.11	4.0	8.09	10.7	37	13	..	..	21	..
II-1-a	10.9	1.11	4.1	8.51	11.3	33	11	..	..	17	..
II-1-b	7.2	1.11	4.0	8.35	11.0	35	10	..	..	16	..
II-1-c	4.8	1.11	4.2	8.64	11.4	37	12	..	..	19	..
I-2	20.7	1.64	3.7	7.71	9.7	..	17	10	8	..	1.7
I-2-a	13.8	1.64	4.0	8.09	10.8	44	16	14	9	29	1.1
I-2-b	9.2	1.64	4.2	8.52	11.0	48	15	..	..	29	..
I-2-c	6.1	1.64	4.2	8.42	11.4	47	12	..	..	23	..
II-2	21.3	2.14	...	7.75	9.7	..	11	..	..	..	..
II-2-a	14.2	2.14	4.0	7.92	10.3	57	15	8	..	35	1.9
II-2-b	9.5	2.14	4.4	8.40	10.1	52	14	8	..	29	1.8
II-2-c	6.3	2.14	4.2	8.36	11.4	55	14	13	..	31	1.1
I-3	23.7	5.9	...	7.31	9 8	..	8	2	2	..	4.0

[a] 100 × a complex/total complexes.

in a future publication. The information obtained from the precipitin tests made it feasible to use BSAI in our experiments. It was decided to prepare a BSAI containing an average of about 5 I atoms per BSA molecule. This figure represents a compromise between a minimally altered and a relatively uniformly labeled BSAI antigen. After iodination, the protein was exhaustively dialyzed against saline at *p*H 7.5, and the solution was analyzed for iodine and nitrogen. The BSAI contained an average of 5.2 I atoms per BSA molecule; it is referred to below as BSA-5I.

Anti-BSA rabbit sera[9] were pooled, and the γ-globulin fraction was largely freed of the other serum proteins by precipitation in 1/3 saturated $(NH_4)_2SO_4$ solution twice, after which it was dissolved in saline at *p*H 7.5. One hundred and eighty-eight ml. of this γ-globulin solution, containing 505 mg. of anti-BSA antibodies, was mixed with 188 ml. of a solution containing 58.7 mg. of BSA-5I. (These proportions were determined in separate tests under the same conditions to be in the zone of maximum precipitation.) This mixture was kept at 4° for about 18 hours. The specific antigen–antibody precipitate was then centrifuged down in the cold, washed once with cold saline, and recentrifuged. Two hundred and fifty mg. of BSA-5I in 17.5 ml. of saline solution was added to the entire precipitate, but after about 18 hours at 4° very little had dissolved. An additional 250 mg. of BSA-5I was then added, and the mixture was mechanically agitated for several hours. The undissolved precipitate (P-A) was centrifuged off, and the supernatant (S-A) was separated. Another 214 mg. of BSA-5I was added to P-A and this mixture was agitated for 24 hours. The remaining undissolved precipitate (about 10 mg.) was centrifuged off and discarded, and the supernatant (S-B) was retained. The two solutions, S-A and S-B were thereafter treated separately. At this stage each of the two solutions contained a rather large excess of antigen, required to dissolve the specific precipitate under the conditions described. As mentioned earlier, it was found possible to prepare solutions of soluble complexes in considerably smaller antigen excess, which was accomplished as follows. S-A and S-B were brought to one-half saturation with respect to $(NH_4)_2SO_4$ and the *p*H was adjusted to 7.8 with dilute NaOH. The precipitates, formed at room temperature for about an hour, were centrifuged down, and were redissolved completely in buffered saline, and the resultant solutions were dialyzed against phosphate buffer, *p*H 7.6 and ionic strength (μ) 0.1. The dialyzed solutions were concentrated by evaporation in dialysis bags at 4° and were then redialyzed against the phosphate buffer. The solutions which resulted were labeled I and II, respectively. Solutions I and II were analyzed for iodine and nitrogen. The factor 6.25 was used to convert from mg. N to mg. protein.

The results listed in Table II indicate that in over-all composition solutions I and II are closely similar. $(AG/AB)_w$ signifies the weight ratio of total antigen to total antibody in a solution. These solutions, containing known amounts of total BSA-5I antigen and total antibody, were the master solutions of soluble complexes used in this study. That part of the BSA-5I not used in the preparation of solutions I and II was concentrated by evaporation in a dialysis bag at 4°, and dialyzed against phosphate buffer, *p*H 7.6, μ 0.1. Solutions of soluble complexes at different and larger antigen excesses were then prepared from I and II by adding to aliquots of them, the appropriate amounts of the latter BSA-5I solution. Ultracentrifugal analyses were carried out at several dilutions at each antigen–antibody ratio studied. For electrophoretic analysis, solutions at various antigen–antibody ratios at a single total protein concentration were prepared, and were then dialyzed for 48 hours at 4° against veronal buffer, *p*H 8.5, μ 0.1.

Materials and Methods.—The BSA utilized throughout this research was the crystalline product obtained from Armour and Co. It was iodinated by the method recommended by Hughes and Straessle.[10] The iodine analyses were performed by the method of Chaney[11] and the nitrogen was determined by Nesslerization. A Beckman Model B spectrophotometer was utilized in the colorimetric assays.

The ultracentrifuge used in this study is an electrically-driven instrument designed and built in this Institute. It employs a two-phase synchronous motor connected directly to the ultracentrifuge rotor. The variable frequency signal driving the motor is generated by an assembly involving a

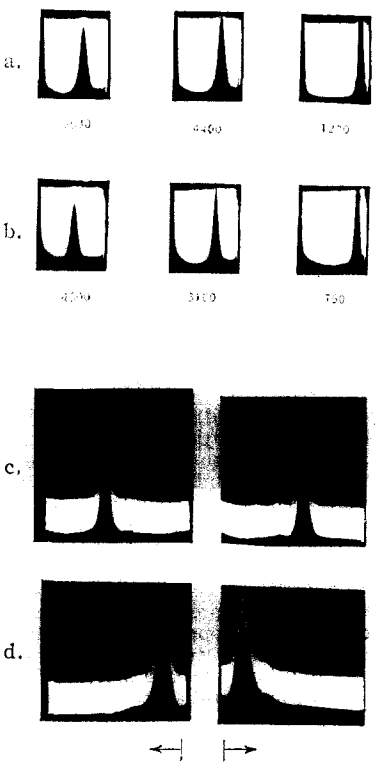
(9) These sera contained an average of about 3 mg./ml. of antibodies to BSA.

(10) W. L. Hughes, Jr., and R. Straessle, THIS JOURNAL, **72**, 452 (1950).

(11) A. L. Chaney, *Ind. Eng. Chem., Anal. Ed.*, **12**, 179 (1940).

calibrated audio oscillator, the output of which is split into two phases and these appropriately amplified. The ultracentrifuge rotor and cells are the standard size analytical equipment supplied by the Specialized Instruments Co., Belmont, California, for its Spinco Model E ultracentrifuge. The optical system is a Philpot–Svensson cylindrical lens–diagonal knife edge schlieren system employing parabolic mirrors instead of schlieren lenses.[12] The temperature of the solution during sedimentation is maintained to within 1° by circulating cold water through coils in the rotor chamber and maintaining hydrogen pressure of about 0.5 mm. in the chamber. The temperature of the solution is measured by means of a calibrated thermocouple situated in the slipstream of the rotor. All of the ultracentrifuge runs were performed at 21 ± 1°, at a rotor speed of 887 ± 2 r.p.s., in phosphate buffer at *p*H 7.6, μ 0.1. The operation of the ultracentrifuge was calibrated by several experiments with 1% solutions of BSA in the phosphate buffer, the sedimentation constant for which was taken as $s^{w}_{20} = 4.05$ *S*.[13]

In the determination of sedimentation constants from diagrams in which the peaks due to the various components were not completely resolved, Gaussian curves[3] were constructed for the major components and the positions of the maxima of these curves were used in the calculations rather than the positions of the maxima as they appeared in the original diagrams. For the evaluation of the areas the original plates were enlarged 13 times and traced. Reference baselines for the diagrams were obtained by ultracentrifuging the buffer itself under conditions identical to those of the protein runs. The dilution factor correction,[14] $(x_t/x_0)^2$, was routinely applied to the areas determined from the sedimentation diagrams.



Ascending. Descending.

Fig. 1.—a, Ultracentrifuge diagrams of BSA-5I; b, ultracentrifuge diagrams of the γ-globulin fraction of rabbit antisera from which the antibodies to BSA were specifically precipitated; c, electrophoresis diagrams of BSA-5I, after 5400 sec. migration; d, electrophoresis diagrams of the γ-globulin fraction, after 5400 sec. migration. The time after full speed was attained is given under each ultracentrifuge pattern. The starting positions of the electrophoresis patterns are indicated by the arrows.

The electrophoresis experiments were performed in a Perkin–Elmer Model 38 Tiselius apparatus. All experiments were performed at a potential gradient of 6.45 volts/cm., for between 7500 to 7800 sec. The *p*H of the veronal buffer was measured at room temperature, and the buffer conductivity, measured at 1.3°, was employed in the mobility calculations.

Results

Starting Materials.—Ultracentrifuge and electrophoresis experiments were performed with the BSA-5I preparation, and with the original γ-globulin fraction from which the antibodies to BSA were subsequently specifically precipitated. Diagrams of these experiments are reproduced in Fig. 1. About 99% of the two preparations migrate ultracentrifugally with the appropriate sedimentation constants under single peaks. Electrophoretically the BSA-5I is about 95% a single component, while of the γ-globulin fraction about 86% migrates under a single peak, the rest of the protein being β- and α-globulins. Since the antibody was subsequently precipitated from this fairly homogeneous γ-globulin fraction, it is considered that any non-specific impurities in the final preparations of soluble complexes were quantitatively insignificant. The most probable contaminant would be non-antibody γ-globulin, and the electrophoresis and sedimentation diagrams to be discussed below reveal no indication of free γ-globulin in the solutions of soluble complexes.

Ultracentrifuge Results.—Several noteworthy features of the schlieren diagrams (examples of which are reproduced in Fig. 2), obtained upon sedimentation of solutions of soluble complexes, may be described, and in the order of increasing sedimentation rate, these features are: (a) the slowest-sedimenting peak, which corresponds to the free BSA-5I antigen[15]; (b) the absence, or presence below detectable limits, of a peak with the sedimentation rate of free γ-globulin; (c) the **a** complex peak, with a sedimentation constant of about 8 *S*, which becomes increasingly prominent in solutions containing a larger excess of antigen, until in the solution with $(AG/AB)_W = 5.9$ it is the predominant complex species; (d) the **b** complex peak, characterized by a sedimentation constant of about 11 to 12 *S*; (e) higher, or **h** complexes, probably consisting of a large number of molecular

(12) S. M. Swingle, *Rev. Sci. Instruments*, **18**, 128 (1947).

(13) S. J. Singer, *J. Chem. Phys.*, **15**, 311 (1947).

(14) T. Svedberg and K. O. Pedersen, "The Ultracentrifuge," Clarendon Press, London, 1940, p. 7.

(15) In the sedimentation diagrams of the solution at a total $(AG/AB)_W = 0.53$, (not illustrated in Fig. 2), the free antigen peak exhibits a sharp spike near its maximum which makes precise area measurements of the albumin peak in this experiment difficult. The behavior of this spike in a number of experiments is analogous to the behavior of certain serum lipid-bearing components studied by Gofman and co-workers.[16] Since this spike becomes decreasingly evident in experiments in larger antigen excess, the substance responsible for the spike was not present in the BSA-5I solution, but must have been in the γ-globulin fraction from which the antibodies were specifically precipitated, and must further have been carried down with the antigen–antibody precipitate. We have not investigated the significance of this observation, nor the possible relationship of the substance to complement or its components. For the purposes of the experiments described in this paper, however, this substance did not significantly affect the results reported.

(16) J. W. Gofman, F. T. Lindgren and H. Elliott, *J. Biol. Chem.*, **179**, 973 (1949).

species not clearly resolved, and varying in over-all composition with variation of $(AG/AB)_W$ of the solution. It is apparent from Fig. 2 that the faster-sedimenting species within the **h** complexes diminish rapidly as the antigen excess increases.

The results of the sedimentation experiments performed with solutions of soluble complexes are summarized in Table II. The first column lists the solution designations: the numbers I and II which are written first in the designations indicate which master solution, I or II, was used to prepare the particular solution. In column 4 appear the sedimentation constants of the free antigen; since the experiments were usually not run much longer than was necessary to obtain satisfactory area measurements under the free antigen peak, the BSA-5I boundary usually did not sediment through a sufficient distance in the cell to permit precise determinations of its sedimentation constants. In columns 7 to 10 are listed the relative areas under the various peaks. The values determined in solutions at higher total protein concentrations are likely to be more accurate. These relative areas were determined as 100 times the ratio of the observed areas of the components to the total area expected in a given ultracentrifuge experiment.[17] This total area was calculated as the product of two terms: the known total protein concentration, and the area per unit protein concentration under a fixed set of optical conditions. The latter term was evaluated from experiments with (a) the BSA-5I preparation; (b) BSA; and (c) various rabbit γ-globulin preparations. Within the experimental errors, the same value of the specific area was obtained for the three materials studied. The relative areas under the free antigen and **a** complex peaks which are listed in columns 7 and 8 are averages of between 4 and 8 exposures in a given experiment, and apart from the dilution factor and the experimental errors the areas did not vary with successive exposures. The areas under the **b** complex peak were often not accurately measurable because of inadequate resolution from the **h** complexes at early stages of an experiment, and because of the proximity of the **b** complex boundary to the rear of the cell at later stages. The areas given for the **b** complex in column 9 are therefore only of qualitative significance. In those experiments in which the apparent area of the **h** complexes was measured, the total observed areas of sedimenting constituents was smaller than the value expected for that solution by about 15 to 20%. This is ascribed to inaccuracies in determining the areas of the **b** and h complexes. In column 11 is given the per cent. of the total complexes constituted by the **a** complex, calculated by dividing the number in column 8 by the difference between 100 and the number in column 7. The ratios of the amount of **a** complex (column 8) to the amount of **b** complex (column 9) are tabulated in column 12. Columns 11 and 12 best illustrate the increasing prominence of the **a** complex in solutions in larger antigen excess.

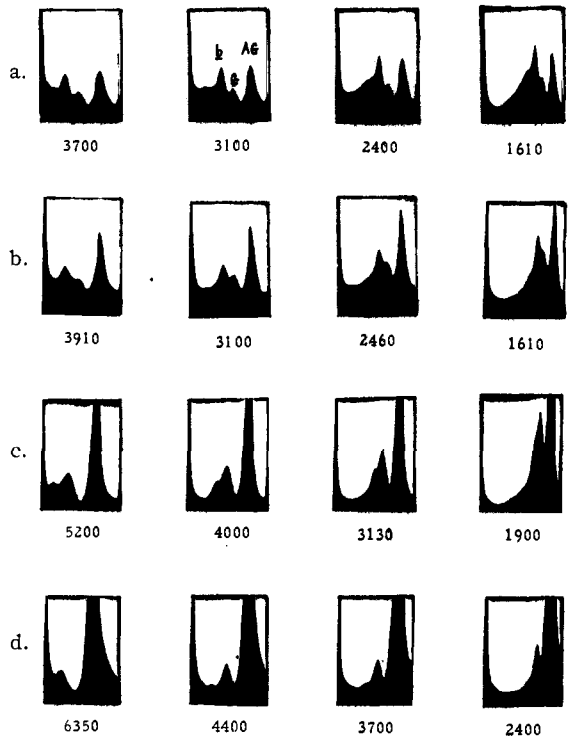


Fig. 2.—Ultracentrifuge diagrams of soluble antigen–antibody complexes, at the following values of $(AG/AB)_W$ and total protein concentrations, respectively: a, 0.56, 18.0 mg./ml.; b, 1.00, 15.7; c, 1.64, 20.7; d, 5.9, 23.7. The time in sec. after full speed was attained is given under each pattern. Sedimentation proceeds to the left.

Electrophoresis Results.—The electrophoresis experiments with the solutions of soluble complexes resulted in the data listed in Table III, and some representative Perkin–Elmer scanning diagrams are presented in Fig. 3. Resolution of the free BSA-5I antigen, the most rapidly-migrating species in solution, was readily achieved in both the ascending and descending limbs of the electrophoresis cell, but the various species of complexes did not separate well under the conditions of our experiments. Only in the solution containing the largest antigen excess studied electrophoretically, E II-2 (corresponding in $(AG/AB)_W$ to solution II-2 examined ultracentrifugally), did the complex peak definitely split into two parts, the faster of which must have been due to the **a** complex. In solutions closer to the equivalence zone than E II-2, the peak representing the complexes was distorted toward the higher mobility side, but no double maximum in the peak appeared. The average mobilities of the complexes which are given in columns 6 and 9 of Table III were calculated from the position of the maximum of the total complex peak, except for solutions E-I-2

(17) Johnston and Ogston[18] have demonstrated that due to the concentration dependence of sedimentation rates of proteins, an artifact may be produced in the apparent relative areas of sedimenting species in a mixture whereby the relative areas of the faster-sedimenting components are decreased, and those of the slower-sedimenting ones are increased. These effects, however, for molecular species of the type we are concerned with in this investigation, are pronounced only at much higher total protein concentrations than those which were examined. The absence of a significant downward trend in the relative areas of free BSA-5I with decrease in total protein concentration at a constant $(AG/AB)_W$ suggests that any such artifacts are within the experimental errors otherwise encountered.

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

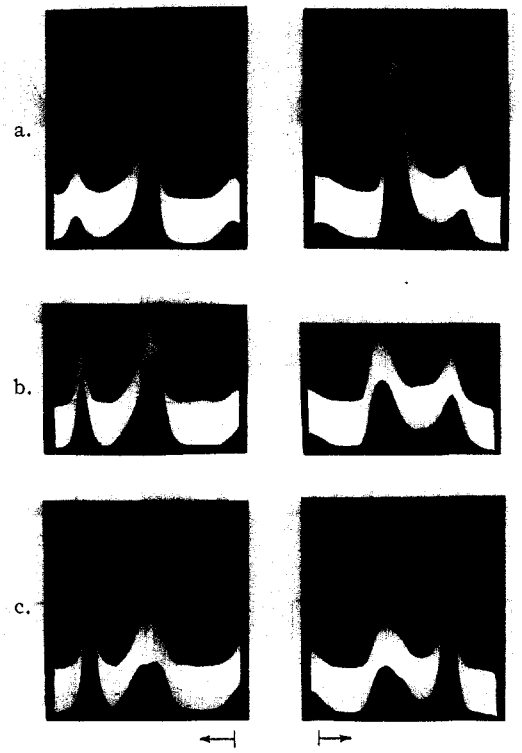


Fig. 3.—Electrophoresis diagrams of soluble antigen–antibody complexes, at the following values of $(AG/AB)_w$, total protein concentration, and times of migration, respectively: a, 0.56, 18.0 mg./ml., 7740 sec.; b, 1.00, 16.1, 7800; c, 2.14, 16.5, 7560. The starting positions are indicated by the arrows.

and E II-2, since no more elaborate measurement seemed justified. It may be noted that, whereas in the ascending limb of each experiment the free antigen peak is completely resolved from the complexes, so that in the region between the peaks the refractive index gradient curve returns to the baseline, in the descending limb the curve between these peaks is elevated from the baseline. The explanation of this effect will be considered below.

TABLE III

ELECTROPHORESIS RESULTS

Soln. no.	$(AG/AB)_w$	Rel. area free BSA-5I ascending	Mobilities[a] Ascending BSA-5I	Ascending complex	Ascending Av.[b]	Descending BSA-5I	Descending complex	Descending Av.[b]
E-I	0.53	8.6	7.3	...	3.4	6.4	...	2.8
E-II	0.56	12.4	7.3	...	3.6	6.6	...	3.0
E-I-1	1.00	28.1	7.4	...	3.7	6.5	...	3.1
E-I-2	1.64	42.6	7.3	...	3.9	6.6	...	3.2
E-II-2	2.14	50.9	7.3	4.8	4.0	6.6	4.4	3.3

[a] In units of 10^{-5} cm.^{2}/volt-sec. All components migrate anodically. [b] Average mobility of complexes, see text.

Recent contributions to the theory and practice of electrophoresis[19] have shown that in order to evaluate most precisely the relative concentrations of non-interacting components from relative areas in electrophoresis diagrams, experiments should be performed at various total protein concentrations (P) and ionic strengths (μ), and the observed relative areas extrapolated to zero P/μ. However, in a system of antigen–antibody complexes such an extrapolation is complicated by the fact that P and μ are variables affecting the equilibrium proportions of the various components, and is therefore of questionable significance. Under the conditions of our experiments the relative areas of the free antigen in the ascending limb are expected to be slightly larger than the true values, but no correction has been made for this small effect in our calculations.

Discussion

Effects of Re-equilibration during Sedimentation and Electrophoresis.—Having presented the experimental results obtained with solutions of soluble antigen–antibody complexes, we are now in a position to discuss a very fundamental aspect of these experiments. The various molecular species in these solutions are in equilibrium with one another. What is the result of effecting a separation of these species in the ultracentrifuge and electrophoresis cells? In particular, what is the quantitative significance to be attached to the various schlieren diagrams recorded in these experiments?

It is immediately apparent that the various components cannot be in very rapidly-adjusted equilibrium with one another. If they were, there would be no resolution into separate peaks in the ultracentrifuge,[20] while in electrophoresis the ascending and descending limbs in a given experiment would show characteristic and pronounced differences.[21] On the other hand, an early experiment which we performed with the un-iodinated BSA–anti-BSA system in the ultracentrifuge indicated that this rate of equilibration among the species in the solution might not be negligibly slow. Two solutions, X and Y, with over all $(AG/AB)_W$ ratios similar to those of solutions I-1 and I-2, respectively, of the present study, were ultracentrifuged after having been prepared at least 24 hours earlier. Another experiment was then performed in which to the solution X was added enough antigen to make up the solution Y, and this preparation was immediately ultracentrifuged. The sedimentation diagrams were the same as those earlier obtained for solution Y, demonstrating that the new equilibrium state was largely attained within the time necessary to mix the solution and begin resolving its components in the ultracentrifuge, which time was less than one hour.

Consideration of the nature of the resolution of the various species in the ultracentrifuge cell and in the two limbs of the electrophoresis cell provides insight into this problem. An analysis of the effects attending the resolution of the species is presented in the appendix to this paper. This analysis demonstrates that certain criteria may be set up to determine whether the effects of re-equilibration significantly affect the quantitative

(19) V. P. Dole, THIS JOURNAL, **67**, 1119 (1945); H. Svensson, *Arkiv Kemi Mineral. Geol.*, **22A**, No. 10, 1 (1946); L. G. Longsworth, *J. Phys. Colloid Chem.*, **51**, 171 (1947).

(20) Reference (14), p. 28.

(21) L. G. Longsworth and D. A. MacInnes, *J. Gen. Physiol.*, **25**, 507 (1942).

interpretation of the schlieren patterns, and these criteria are utilized in the following sections.

The Amount of Free Antigen.—The major feature of the experiments which is used extensively in a quantitative manner in the following sections is the relative area of the free antigen peak. In view of the discussion presented in the last section and in the appendix, two factors may be cited to justify the conclusion that the free antigen area determined from the ultracentrifuge diagrams and from the ascending electrophoresis diagrams represents the true relative concentration of the free antigen in the solution being examined: (a) the absence of any additional refractive index gradients between the free antigen peak and the **a** complex peak; and (b) the agreement of the areas obtained by these two independent methods.

Because of the better resolution and the more precise location of the schlieren diagram baseline in the ascending electrophoresis patterns than in the ultracentrifuge patterns, and for other reasons mentioned in the appendix, the free antigen concentrations determined from the former patterns are somewhat more reliable. These concentrations determined electrophoretically are usually slightly smaller than those obtained from the sedimentation experiments: this may be due to experimental error, or to the fact that the two types of experiments were performed in somewhat different buffers and at different temperatures.

The a Complex.—Since the **a** complex so clearly predominates over other complexes in large antigen excess, it must be the richest antigen-containing complex possible in this system. The constitution of this complex is therefore of primary importance for the problem of the valence of the antibodies, for in the **a** complex the antibody molecule is binding as many antigen molecules as its valence permits.

The sedimentation constants of the **a** and **b** complexes in a variety of solutions are given in columns 5 and 6 of Table II. In order to obtain sedimentation constants characteristic of these species and independent of concentration effects, these constants were plotted as functions of the effective concentrations of the solutions through which the complexes sedimented. The effective concentration in a particular solution was taken, for the **a** complex, as the sum of the concentrations of the **a** complex and the free antigen, determined from the relative areas in the sedimentation pattern; for the **b** complex, the sum of the concentrations of the **a** and **b** complexes and the free antigen was utilized. Johnston and Ogston[18] have shown with mixtures of various proteins that such effective concentrations can be used for the extrapolation of sedimentation constants provided that the concentration dependences of the sedimentation constants of the individual species in the mixture are not too pronounced. Figure 4 illustrates these plots for the **a** and **b** complexes. The straight lines drawn through the experimental points were determined by least-squares analyses, and the extrapolated values of s^{w}_{20} are 8.7 and 12.0 S for the **a** and **b** complexes, respectively.

For the present let us restrict our attention to the

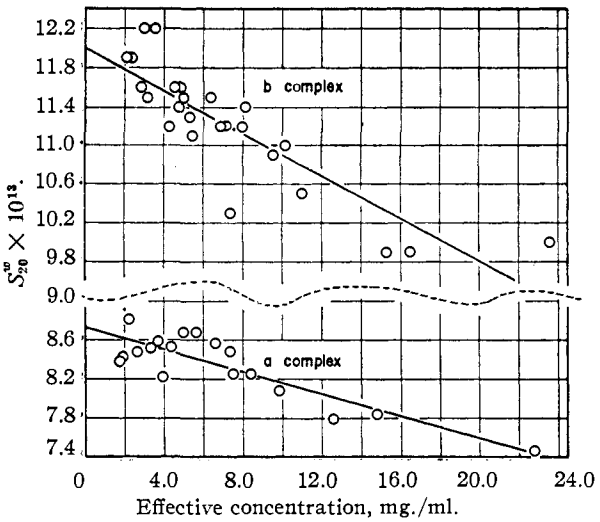


Fig. 4.—Sedimentation data for the **a** and b complexes.

a complex. Since the average shape of the molecules of the **a** complex is unknown, the molecular weight, M, of the **a** complex cannot be directly determined from its sedimentation constant. Instead, various possible combinations of M and f/f_0, the frictional ratio, exist which are compatible with a given s^{w}_{20}, according to the Svedberg equation

$$M = \left[\frac{s(f/f_0)6\pi\eta N(3\bar{V}/4\pi N)^{1/3}}{1 - \bar{V}\rho}\right]^{3/2}$$

η is the viscosity and ρ the density of the buffer solvent, $\bar{V}$ the partial specific volume of the solute, and N is Avogadro's number. From the known values of M for BSA and rabbit γ-globulin, the molecular weights expected for various species of complexes can be calculated, and these are listed in column 3 of Table IV. In order for the **a** complex to have the molecular weight given for any one of these species, it must be characterized by the adjacent f/f_0 value in column 4. Thus, if the **a** complex molecule were constituted of 3 antigen molecules and 1 antibody (3AG:1AB), it would have an f/f_0 value of 2.1. This value appears to be too large to be compatible with any possible structure containing 3 BSA molecules attached to the same γ-globulin molecule. For this reason, and from the results of quantitative considerations to be discussed below, it is improbable that the **a** complex is the 3AG:1AB species, or one still richer in antigen. Up to this point, however, it is not possible to decide whether the **a** complex is largely the 2AG:1AB or the 1AG:1AB species,

TABLE IV

MOLECULAR WEIGHT DATA

Species	$s^{w}_{20} \times 10^{13}$	M	f/f_0
BSA (AG)	4.5	70,000	1.3
Rabbit (AB)	7.0	160,000	1.5
a Complex	8.7	230,000 (1AG:1AB)	1.5
		300,000 (2AG:1AB)	1.8
		370,000 (3AG:1AB)	2.1
b Complex	12.0	390,000 (1AG:2AB)	1.6
		460,000 (2AG:2AB)	1.8
		530,000 (3AG:2AB)	1.9
		600,000 (4AG:2AB)	2.1

with the appropriate value of f/f_0, or a mixture of roughly equal proportions of the two types, having coincidentally the same sedimentation constant.

The last possibility, that the **a** complex is a mixture of the two species, may be excluded as follows. Ultracentrifugal examination reveals that the **a** complex constitutes about 33% of all of the complexes in solution II-2, while electrophoretically, it is found that 40% of the area of all of the complexes is accounted for under the **a** complex peak in solution E II-2. However, 1AG:1AB and 2AG:1AB complexes should have markedly different electrophoretic mobilities,[22] and if the **a** complex were a mixture of about equal amounts of the two types, which sedimented at the same rate but were resolved electrophoretically, the relative area under the **a** complex peak in electrophoresis would have been about $^1/_2$ of that found under the **a** complex peak in sedimentation. The fact that all of the **a** complex peak observed ultracentrifugally can be accounted for under a single peak electrophoretically makes it unlikely that the **a** complex is a mixture of about equal amounts of the two types of complexes, but is rather largely one or the other.

The following considerations demonstrate that the **a** complex cannot be the 1:1 species. If the amount of free antigen in a given solution (taken from the ascending electrophoresis pattern) is subtracted from the total antigen present, the result is the total amount of antigen bound in all of the complexes in that solution. This, divided by the total antibody (practically all of which is bound up in complexes), and multiplied by the molecular weight ratio 160,000/70,000, gives $\overline{(AG/AB)}_{N,B}$, the *average* number of antigen molecules bound per antibody in *all* of the complexes in the particular solution. The results of these calculations are presented in Table V. The values of $\overline{(AG/AB)}_{N,B}$, listed in column 4 of this table, increase regularly to well above unity as the antigen excess is increased. The sedimentation patterns show that in solution I about 5% of all of the complexes is the **a** complex, whereas in solution II-2, it is about 33%, the other complexes in these solutions being characterized by smaller antigen–antibody ratios. The **a** complex must therefore obviously be richer in antigen than the 1AG:1AB species, for $\overline{(AG/AB)}_{N,B}$ would otherwise not increase to a value above unity.

(22) Calculations were made, according to the method described by Abramson, Moyer and Gorin,[23] for the mobilities to be expected for various types of complexes under the electrophoretic conditions which we employed. The charges carried by the complexes were taken as the sum of the charges of their constituent molecules. The latter charges were evaluated from the descending mobilities of -6.5×10^{-5} and -1.6×10^{-5} cm./sec./volt/cm. for BSA and rabbit γ-globulin, respectively. The appropriate frictional ratios were taken from column 4 of Table IV of this paper. The model of an unhydrated long cylinder was employed and a uniform distribution of charge on the surface of the complex was assumed. 1AG:1AB and 2AG:1AB complexes were thus accorded mobilities of -4.1×10^{-5} and -5.3×10^{-5} cm./sec./volt/cm., respectively. Because of the restrictive assumptions involved in making these calculations, the precise values of these theoretical mobilities are of questionable significance, but the theory does indicate that the two types of complexes should have distinctly different mobilities.

(23) H. A. Abramson, L. S. Moyer and M. H. Gorin, "Electrophoresis of Proteins," Reinhold Publishing Corp., New York, N. Y., 1942, p. 152.

TABLE V

COMPOSITION OF ANTIGEN–ANTIBODY COMPLEXES

Soln. no.	Total antigen, %	Free antigen, %	$\overline{(AG/AB)}_{N,B}$	$\overline{(AG/AB)}'_{N,B}$ $a = 2:1$	$\overline{(AG/AB)}'_{N,B}$ $a = 3:1$
E-I	34.7	8.6	0.92	0.87	0.85
E-II	35.8	12.4	0.82	.77	.74
E-I-1	50.0	28.1	1.01	.88	.81
E-I-2	62.2	42.6	1.19	.92	.74
E-II-2	68.1	50.9	1.24	.96	.74

Extension of this quantitative approach one more step provides further evidence that the **a** complex is not the 3AG:1AB species. Using the amounts of the **a** complex in each solution determined ultracentrifugally, the antigen and antibody in the **a** complex can be subtracted from the total antigen and antibody, respectively, which are bound in all of the complexes, assuming that the **a** complex is either the 2AG:1AB or the 3AG:1AB species. This permits calculation of values of $\overline{(AG/AB)}'_{N,B}$, the average number of antigen molecules bound per antibody molecule in all of the complexes in that solution *other than the* **a** *complex*. Assuming that the **a** complex is the 3AG:1AB species results in the values of $\overline{(AG/AB)}'_{N,B}$ given in column 6 of Table V, which decrease as the antigen excess is increased, whereas the values in column 5, with the **a** complex taken as the 2AG:1AB species, increase slightly with increased antigen excess. The latter trend is in accord with mass action considerations, whereas the former is not.

To summarize the results of this section: we have demonstrated that the **a** complex: (a) is the richest antigen-containing complex capable of being formed by the antibodies in this system; (b) cannot be the 3AG:1AB, or any complex species with higher molecular weight; (c) cannot be a mixture of roughly equal amounts of 2AG:1AB and 1AG:1AB complexes; and (d) cannot be largely the 1AG:1AB species. The only remaining possibility is that the **a** complex is largely the 2AG:1AB species. Furthermore all the data reported in this paper are compatible with this conclusion. It follows that the antibodies in this system are largely bivalent.

The maximum amount of univalent antibody that could be tolerated within the framework of these results is probably about 20% of the total antibody if the 1AG:1AB species were coincidentally to have the same sedimentation constant as the 2AG:1AB complex. Otherwise, ultracentrifugal resolution of the two types of complexes would permit the detection of about 5% of univalent antibodies.

It should be recalled that the antibody studied in this investigation is a rabbit antibody precipitated with its slightly modified homologous antigen. It is conceivable that the course of immunizaton might have a bearing on the possible production of univalent antibodies. It is also possible that univalent antibodies were present in the original antisera used in this study, but that they were not extensively carried down in the antigen–antibody precipitate. It is felt, however, that the nature of the antigen and the antibodies utilized in this investigation permits considerable generality to be

accorded to the conclusion that precipitating rabbit antibodies are largely bivalent.

Since we have demonstrated that the **a** complex is largely the 2AG:1AB species, it follows from the sedimentation data given in Table IV that the value of f/f_0 for this complex is 1.8. This large value indicates that the **a** complex molecule is considerably asymmetric, and suggests that the antigen molecules are situated near the opposite poles of the elongated antibody molecule. If the departure of f/f_0 from the value of unity for the different molecules involved in this study is ascribed solely to asymmetry, neglecting the effects of solvation, and the model of a prolate ellipsoid of rotation is applied to each, then the axis ratios of such ellipsoids are found to be about 5, 9, and 19, for BSA, rabbit γ-globulin, and the **a** complex molecules, respectively.[24] The last number is the sum of the axis ratios of 2 antigen molecules and 1 antibody, suggesting that these three molecules are situated in a linear array in the **a** complex. The unknown contribution of solvation to the values of f/f_0, however, renders this conclusion somewhat less certain.

The b Complex.—Consideration of the law of mass action applied to the sedimentation diagrams of Fig. 2 indicates that the **b** complex is characterized by a smaller antigen-antibody ratio than the **a** complex. Although no definitive set of arguments can be submitted to define the composition of the **b** complex, all of the information available is compatible with the proposal that it is the 3AG:2AB species. If we therefore accord the **b** complex the molecular weight 530,000, and utilize the extrapolated value of $s^{0}_{20} = 12.0\ S$ the complex is characterized by an average value of $f/f_0 = 1.9$. This value implies that the molecules making up the **b** complex are not all arranged linearly, since f/f_0 would be about 2.4 for such a structure. Indeed, it is reasonable to expect that, since the antigen is probably multivalent, the two antibody molecules of the **b** complex could be bonded to the central antigen molecule in a considerable number of equally probable configurations. This would result in an average value of f/f_0 smaller than that which would be expected for a linear array of the 3 antigen and 2 antibody molecules, but larger than the value found for the linear array of 2 antigen and 1 antibody molecules. The observed results are in accord with these considerations.

Additional experiments may be proposed in order to test and extend the conclusions reached in this paper. We have performed electrophoresis experiments with the BSA-5I- anti-BSA system under a single set of conditions, and it would be of considerable interest to examine this system in buffers of different ionic strengths and pH values with the object, among others, of achieving better electrophoretic resolution among the complexes. If satisfactory resolution of the **a** complex from the other complexes can be accomplished, it should be possible to withdraw samples from the electrophoresis cell for analysis, and thus more directly establish the composition of the **a** complex. Obviously, many other antigen–antibody systems should be quantitatively investigated by the physical chemical methods we have employed in this study, not only to investigate the problem of antibody valence, but also the problem of antibody structure, as revealed by the analysis of the frictional ratios attributable to the **a** and **b** complexes in a given system.

Studies utilizing these methods should also be useful in the detection of univalent antibodies possibly present in antisera, but which might not be extensively coprecipitated with the antigen and bivalent antibodies. Under appropriate conditions, ultracentrifugal resolution of a complex of composition 1AG:1AB might be achieved. Such a complex would probably be most readily detectable if the antigen were to have a molecular weight and a sedimentation constant close to those of the antibody.

The Free Energy Change of the Antigen–Antibody Reaction.—In this investigation we have obtained information concerning the amounts of free BSA-5I antigen present in equilibrium with antigen–antibody complexes in solutions containing known total amounts of antigen and antibody. This information has been used to determine the valence of the antibodies in the system. These data, however, are also of considerable interest in connection with the determination of the equilibrium constants and the free energies characterizing the reaction of the antigen and antibody. The treatment of this problem will appear in a future publication.

Acknowledgement.—We wish to express our gratitude to Professors Linus Pauling and Verner Schomaker for their many useful suggestions and criticisms in the course of this study. Professor Schomaker, in particular, contributed many of the ideas contained in the section on the effects of the rates of equilibration of the complexes upon the sedimentation and electrophoresis diagrams. The competent assistance of Mr. J. Banovitz is also gratefully acknowledged.

Appendix

Effects of Re-equilibration during Sedimentation and Electrophoresis.—It is not our object to present a general and rigorous theory for the effects of moderate rates of re-equilibration in a multi-component system upon the sedimentation and electrophoresis patterns of the system. Instead, the discussion is restricted to the special case that is applicable to the BSA-5I–anti-BSA system under the conditions studied in this investigation. Our purposes are: (a) to achieve a general understanding of the nature of these effects; (b) to set up criteria for the quantitative use of the schlieren diagrams; and (c) to indicate qualitatively the reasons for the observed small differences between the ascending and descending electrophoresis patterns.

Let us first consider what happens during sedimentation. We have observed that the rapidly-sedimenting h complexes are prominent in antigen–antibody solutions in low antigen excess, whereas the slowest-sedimenting complex, the **a** complex, predominates in solutions in large antigen excess. Thus, the more rapidly sedimenting species must be richer in antibody than the **a** complex. As the more rapidly sedimenting boundaries move through the cell, therefore, those molecules of the other complexes which are left behind undergo a variety of reactions to adjust to their new environments.

Since our present interests are mainly in the **a** complex and the free antigen, the effects of these reactions may be appropriately illustrated by considering an idealized equilibrium system containing substantially only the three species $(AG)_3(AB)_2$ (the **b** complex), $(AG)_2(AB)$ (the **a** complex),

(24) Reference (14), p. 41.

and AG (free antigen). These species have the sedimentation constants $s_b > s_a > s_A$, and upon sedimentation they form the sharp boundaries **b**, **a**, and **A**, respectively. That part of the system behind **b** no longer has the original composition, and tends toward a new state of equilibrium. Behind **a** no reaction occurs, only AG being present, but between **a** and **b**, the original $(AG)_3(AB)_2$ having sedimented away, disproportionation of $(AG)_2(AB)$ takes place

$$2(AG)_2(AB) \xrightarrow{R} (AG)_3(AB)_2 + AG$$

R is the over-all rate constant of this reaction. The additional AG so produced sediments along with the AG originally present. Consider the excess AG produced at time t_1 at the position P a distance x ahead of **A** (Fig. 5a). At some later time t_2 that AG produced at P will have been overtaken by **a** and will be found in the interzone **a** − **A** (Fig. 5b).

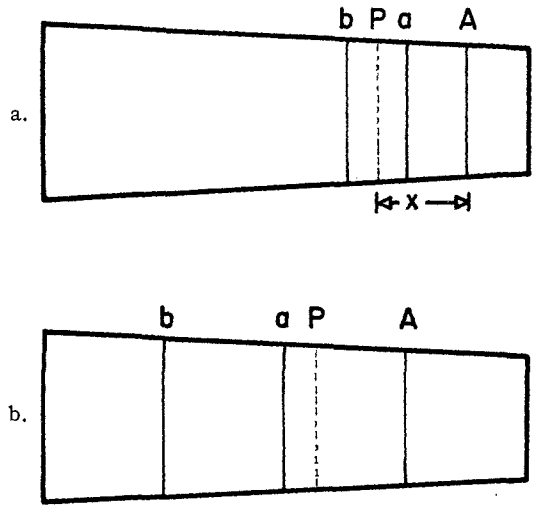


Fig. 5.—Boundary positions for the idealized three-component system in the ultracentrifuge cell. See appendix for details.

The total excess of AG found at position P, ΔAG, is simply the amount produced at P in the time interval between the arrivals of **b** and **a** at P, namely

$$\Delta AG = \int_{t(x,b)}^{t(x,a)} R(x)dt = \overline{R(x)}\left\{\frac{x}{s_a - s_A} - \frac{x}{s_b - s_A}\right\} = \frac{\overline{R(x)}\, x(s_b - s_a)}{(s_a - s_A)(s_b - s_A)}$$

Here $\overline{R(x)}$ is the effective average rate of reaction. It follows that $d(\Delta AG)/dx$ is positive (and equal to the constant $\bar{R}(s_b - s_a)/(s_a - s_A)(s_b - s_A)$ if $\bar{R}$ is constant) and that the schlieren curve in the region **a** − **A** will be elevated from the baseline correspondingly. If, on the other hand, the schlieren curve in this region does return to the baseline, then the effects of re-equilibration are negligible, and unambiguously, *the area under the free antigen peak corresponds to the concentration of free antigen present in the original solution.* With more than three species the situation is more complicated, but the same conclusion holds.

For the system BSA-5I-anti-BSA at *p*H 8.5, the electrophoretic mobilities of the various species have the order $-\mu_A > -\mu_a > -\mu_b > 0$. As a result, the ascending limb in electrophoresis exhibits a distribution of species which is similar to that produced in the ultracentrifuge, and the considerations discussed above apply, with only minor changes. The effects of re-equilibration are probably even less significant in the ascending limb in electrophoresis than in the ultracentrifuge. The electrophoresis experiment is performed near 0°, whereas sedimentation is carried out at about 20°. The rates of the re-equilibration reactions should therefore be slower in electrophoresis. Furthermore μ_a and μ_b are not very different, and the term $(\mu_a - \mu_b)/[(\mu_A - \mu_a)(\mu_A - \mu_b)]$ should be less important than the corresponding sedimentation term. For these two reasons, the ascending electrophoresis pattern should be more reliable than the sedimentation pattern, other things being equal.

In the descending limb of the electrophoresis cell, however, a markedly different distribution of components exists for this system. Between the free antigen boundary and the **a** complex boundary, *all* of the complexes are present in a region deprived of the free antigen originally in the solution. These complexes will then undergo reactions to produce free antigen. An analysis of the results by methods similar to those applied to the ascending limb has been made, but since the descending patterns have not been used quantitatively in this paper, and the effects of re-equilibration are involved, the analysis will not be detailed here. It is possible by this means to explain the experimental observation that in the descending limb, but not in the ascending, the schlieren curve between the free antigen peak and the a complex peak is somewhat elevated from the baseline.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY AND THE DEPARTMENT OF PHYSICS, KANSAS STATE COLLEGE]

Autoxidation of Ketones. II. Di-*n*-propyl Ketone[1]

BY DEXTER B. SHARP, STUART E. WHITCOMB, LEO W. PATTON AND A. DWIGHT MOORHEAD

Liquid di-*n*-propyl ketone has been oxidized by molecular oxygen to propionic and *n*-butyric acids, in the absence of inorganic catalysts. A small amount of water and propionaldehyde also was obtained, but no *n*-butyraldehyde was isolated. The oxidations were followed by chemical analyses. The peroxide content increased very slowly, but the acid content increased rapidly after the induction period. Infrared spectra were obtained periodically during the runs, and changes in spectra with oxidation time are reported. The results provided further evidence of the α-position activation by the carbonyl group during autoxidations of ketones.

Introduction

In a previous paper,[2] the autoxidation of diisopropyl ketone to an α-hydroperoxide, acetone and isobutyric acid was reported. Similar studies have been made of the autoxidation of di-*n*-propyl ketone. Paquot[3] reported that di-*n*-propyl ketone was oxidized by oxygen to a mixture of *n*-butyric acid, propionic acid and smaller amounts of heptanedione-3,4 and *n*-butyraldehyde. However, he used nickel phthalocyanine, which is known to accelerate autoxidation processes. It seemed logical that the exclusion of such catalysts might provide more reliable information about the course of oxidation.

A multiple investigation of the autoxidation was carried out. Inorganic catalysts were excluded

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(2) D. B. Sharp, L. W. Patton and S. E. Whitcomb, THIS JOURNAL, **73**, 5600 (1951).

(3) C. Paquot, *Bull. soc. chim.*, **12**, 450 (1945).