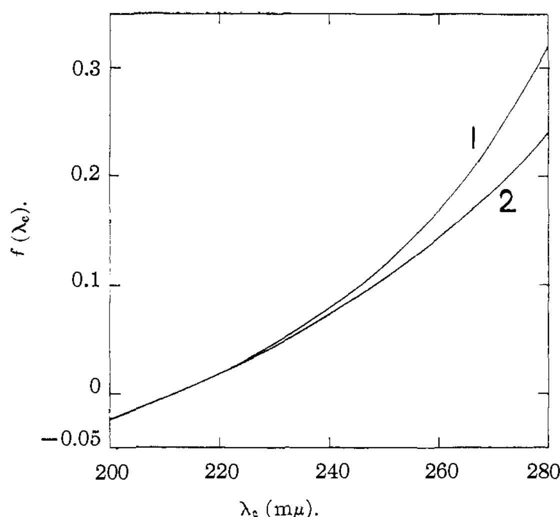

Fig. 4.—A plot of the function $f(\lambda_c)$. In curve 1 equations 1 and 2 were equated at 350 and 650 mμ; in curve 2 they were equated at 450 and 550 mμ.

accidental result of the action of hydrophobic forces. As pointed out above, peptide groups removed from the surrounding water by the action of these forces will seek to form whatever hydrogen bonds they can form without interfering with the need to keep non-polar groups out of contact with the water. It is entirely reasonable that α-helical regions can sometimes be formed in this way.[38]

(38) It has recently been demonstrated that myoglobin[39] and hemoglobin,[40] in water-containing crystals, have about two thirds of their peptide links participating in the formation of α-helices. If the conclusions of the present paper are correct, then the helix content of these proteins appears exceptionally large. It is also possible (but not considered likely) that the molecular conformation in water-containing crystals of proteins may often differ from the conformation in aqueous solution.

(39) J. C. Kendrew and co-workers, *Nature*, **185**, 422 (190).

(40) M. F. Perutz and co-workers, *ibid.*, **185**, 416 (1960).

Our over-all conclusion is that globular proteins have an intrinsic tendency to form α-helices only in solvents which have a large content of an organic substance. The chief proteins which possess extensive helical regions in water are the non-globular proteins which form long rod-shaped particles in solution, such as myosin and related proteins. Why they prefer this structure is an intriguing question.

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Appendix

Calculation of b_0 from the Parameters of Equation 2.—Some of the data used above were originally reported in terms of equation 2 rather than equation 1. Since each equation contains two variable parameters, conversion from one equation to the other can be performed by stipulating that $[m']_D$ be identical at two wave lengths. The resulting relation for b_0 is

$$b_0 = Af(\lambda_c) = [m']_D(34.727 - 10^{10}\lambda_c^2)f(\lambda_c) \quad (3)$$

where λ_c is given in cm., and the function of $f(\lambda_c)$ depends not only on the value of λ_c but also on the two wave lengths at which the two equations are taken as identical. The value of a_0 is obtained from equation 1 by using the given value of $[m']_D$ and the value of b_0 calculated by equation 3.

If any significance is to be attached to this calculation, it is necessary that the result is independent of the choice of the two reference wave lengths. Accordingly, we have plotted in Fig. 4 the function $f(\lambda_c)$ of equation 3 for two choices of the reference wave lengths, one pair being near the extremes of the range usually used, the other pair being closer together. The figure shows that the conversion from one equation to the other is valid for $\lambda_c < 250$ mμ. If equation 2 is accurately obeyed with a larger value of λ_c, then a plot according to equation 1 cannot be linear. Alternatively, equation 2 may not be applicable, the apparent applicability reported being the result of choice of a relatively narrow range of wave length. The second explanation probably applies to the values of λ_c in excess of 250 mμ reported by Schellman.[14] He used a wave length range of 436 to 589 mμ.

Conversion from equation 2 to equation 1 has been used in the present paper only for $\lambda_c < 250$ mμ.

[CONTRIBUTION NO. 1595 FROM THE STERLING CHEMISTRY LABORATORY, YALE UNIVERSITY, NEW HAVEN, CONNECTICUT]

Physical Chemical Studies of Soluble Antigen–Antibody Complexes. XII. The Free Energy Change in the Reaction between Bovine Ribonuclease and its Rabbit Antibodies

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By means of electrophoretic studies on mixtures of bovine pancreatic ribonuclease and its purified rabbit antibodies, the intrinsic equilibrium constant and standard free energy change characterizing the specific interaction have been determined as $(4 \pm 2) \times 10^3$ l./mole and -4.5 ± 0.2 kcal./mole, respectively, in acetate buffer, pH 5.68, Γ/2 0.1, at 1°. These values are remarkably similar to corresponding quantities obtained in other antigen–antibody reactions involving precipitating rabbit antibodies, and the significance of this result is discussed.

In previous studies from this Laboratory, thermodynamic quantities characterizing the reactions of protein antigens (Ag) and their rabbit antibodies (Ab) have been obtained for the antigens bovine serum albumin (BSA)[3] and ovalbumin (OA).[4] It was found that the free energy, enthalpy and entropy changes were each very similar for the two unrelated systems. In order to extend and generalize these observations, we have now determined the free energy change of the reaction between bovine pancreatic ribonuclease (RNase)

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(2) Department of Biological Chemistry, Marischal College, University of Aberdeen, Aberdeen, Scotland.

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

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

and its rabbit antibodies. RNase is a protein significantly different from BSA and OA in at least two gross respects: its isoelectric point is much higher,[5] and thus its electric change properties are different; and its molecular weight is unusually small, and therefore the steric interactions in complexes containing RNase and its Ab could conceivably be atypical. In addition to these reasons for utilizing this protein, RNase has been extensively studied with respect to its molecular and chemical properties,[6] as well as its antigenic properties.[7,8]

The method for obtaining the free energy change, as developed and described in our earlier studies,[3,4] was to determine by electrophoresis the concentration of free RNase in homogeneous solutions containing known amounts of total RNase and total Ab in Ag excess.

Materials and Methods

Proteins.—Crystalline bovine pancreatic RNase, Armour lot no. 381-059, was used without further purification throughout this work. Although this material contains at least the two fractions RNase-a and RNase-b, these are antigenically indistinguishable.[7,8] Normal rabbit γ-globulin was prepared by repeated precipitation of the γ-globulin fraction from normal rabbit sera with 37% saturated ammonium sulfate (SAS).

Immunization of Rabbits.—Rabbits were injected subcutaneously with doses of 10-15 mg. of RNase mixed with Freund's complete adjuvant (available from Difco Labs, Detroit, Michigan). Two injections, spaced three weeks apart, were usually sufficient to induce a strong immune response which lasted as long as 4 to 6 months. Additional booster injections were given whenever the Ab level decreased appreciably. High titer antisera were pooled and the γ-globulin fraction was precipitated with 40% SAS and was stored at 5° until needed.

Preparation of Soluble Ag-Ab Complexes.—In our earlier studies[3,4] solutions of Ag-Ab complexes were prepared without ever isolating the Ab, by forming an Ag-Ab precipitate at the equivalence point and then, after the precipitate was freed of inert protein, dissolving the precipitate in an excess of the Ag. The manipulations following these steps required the dialysis of the Ag-Ab solutions into electrophoretically-suitable buffers without change of relative protein composition. In the present work, however, it was found that the dialysis casing was partially permeable to RNase, and the same method could not be used. Instead, anti-RNase Ab was first prepared in a pure state; separate solutions of RNase and Ab were dialyzed into the appropriate buffer; the concentration of each solution was determined by differential refractometry with a Brice-Phoenix instrument; mixtures of the two solutions were prepared by weight and were then directly examined electrophoretically.

Anti-RNase Ab was purified by the method described by Singer, Fothergill and Shainoff.[9] Two preparations were used in this study, designated A and B in Table V of ref. 9. Only about 70% of the purified Ab preparation was precipitable by RNase, but this most likely reflects the attainment of an equilibrium in the Ag-Ab reaction, rather than the presence of 30% inactive protein. This is indicated by the observation[9] that the maximum amount of Ab precipitated increased with increasing total protein concentration of the Ag and Ab solution. Similar incomplete precipitation of pure Ab has been observed in other Ag-Ab systems in which the Ag valence is small.[10]

Electrophoresis.—All experiments were performed at 1° in acetate buffer, pH 5.68, $\Gamma/2$ 0.1 for 9000 sec. at a field strength of 8.53 volts/cm. The buffer was chosen because it permitted adequate electrophoretic resolution of the system without being sufficiently acid to produce appreciable dissociation of the Ag-Ab bonds in this system, as determined by precipitin tests. (In veronal-NaCl buffer at pH 8.5, in which the BSA and OA systems were investigated, the electrophoretic separation of RNase and Ab is poor.) The total protein concentration was close to 15 mg./ml. A Perkin-Elmer model 38A Tiselius apparatus equipped with interference optics was used. The Rayleigh fringe patterns from the ascending limb were analyzed according to the method of Longsworth,[11] using a Gaertner Toolmaker's Microscope, to obtain the fractional number of fringes, N, characterizing the free RNase boundary in a particular solution. The total relative refractive increment, ΔD, of the solution was separately determined in a differential refractometer. In order to obtain the apparent per cent. free RNase from the ratio $N/\Delta D$, and simultaneously to correct this figure for any electrophoretic boundary anomalies, separate calibration experiments were carried out with several known mixtures of RNase and normal rabbit γ-globulin under conditions as similar as possible to those used for the RNase-Ab mixtures. From measurements on the ascending patterns of these experiments, a least-squares straight line was obtained for the ratio $N/\Delta D$ as a function of the known per cent. RNase, which was then used to obtain the per cent. free RNase from the $N/\Delta D$ data for the RNase-Ab solutions.

Ultracentrifugation.—The RNase-Ab solutions were recovered from the electrophoresis experiments and were examined at concentrations close to 8 mg./ml. in a Spinco Model E Analytical Ultracentrifuge operating around 20° at a rotor speed of 50,740 r.p.m., using 30 mm. cells. The apparent per cent. free RNase was determined from the area under the free RNase peak in the ultracentrifuge pattern, and from ΔD for the solution as recovered from the electrophoresis cell by methods previously described.[12] No corrections were made for the Johnston-Ogston effect.[13]

Results

Electrophoresis.—The results of six RNase-normal γ-globulin calibration experiments are given in Table I and of four RNase-Ab experiments in Table II. Representative fringe patterns of comparable experiments of the two types of mixtures are shown in Fig. 1. At pH 5.68, RNase migrates more rapidly than γ-globulin. Only two peaks appeared in the ascending and descending patterns with both types of mixtures. The faster-migrating boundary in the RNase-Ab solutions had the same mobility as the RNase boundary in

TABLE I

ELECTROPHORESIS OF MIXTURES OF RNASE AND NORMAL GAMMA GLOBULIN

Soln.	ΔD[a]	N[b]	$N/\Delta D$	RNase, % Anal.[c]	Calcd.[d]
1	2.905	30.43	10.48	51.0	51.4
2	2.912	26.32	9.04	44.6	43.5
3	2.925	24.48	8.37	39.9	39.9
4	2.910	22.32	7.67	34.9	36.0
5	2.924	19.24	6.58	30.0	30.1
6	2.921	16.29	5.58	24.9	24.6

[a] Relative refractive increment of solution in arbitrary instrument units. [b] Fractional number of fringes in ascending RNase boundary. [c] Known from proportions of RNase and γ-globulin solutions mixed. [d] Determined from equation % RNase = 5.463 $N/\Delta D$ − 5.850, the least-squares straight line obtained from numbers of columns 4 and 5.

(5) E. A. Anderson and R. A. Alberty, *J. Phys. Colloid Chem.*, **52**, 1345 (1948).

(6) C. H. W. Hirs, W. H. Stein and S. Moore, *J. Biol. Chem.*, **221**, 151 (1956).

(7) B. Cinader and J. H. Pearce, *Brit. J. Exptl. Pathol.*, **37**, 541 (1956).

(8) R. K. Brown, R. Delaney, L. Levine and H. Van Vunakis, *J. Biol. Chem.*, **234**, 2043 (1959).

(9) S. J. Singer, J. E. Fothergill and J. R. Shainoff, THIS JOURNAL, **82**, 565 (1960).

(10) F. A. Pepe and S. J. Singer, *ibid.*, **81**, 3878 (1959).

(11) L. G. Longsworth, *Anal. Chem.*, **23**, 346 (1951).

(12) S. J. Singer and D. H. Campbell, THIS JOURNAL, **74**, 1794 (1952).

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

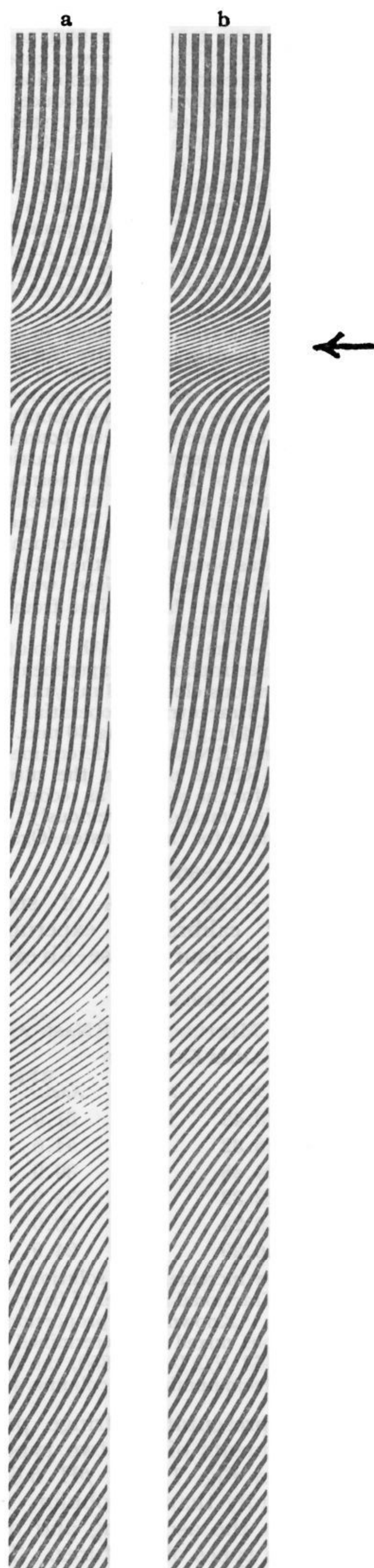

Fig. 1.—Rayleigh interference patterns of the ascending limbs of electrophoresis experiments on: (a) a mixture of RNase and normal rabbit γ-globulin (solution 3, Table I); and (b) a mixture of RNase and rabbit anti-RNase antibody (solution 8, Table II); in acetate buffer, *p*H 5.68 $\Gamma/2$ 0.1. Migration is upwards in the figure; the arrow indicates the boundary due to free RNase.

the control mixtures, while the slower-moving boundary (the reaction boundary)[14] was very similar to the γ-globulin boundary in the control mixtures. The low molecular weight of the RNase molecule makes its contribution to the mobility of Ag–Ab complexes relatively small, and therefore in this system these complexes do not migrate much more rapidly than free γ-globulin. The per cent. by weight of RNase which was found in Ag–Ab complexes, which is the difference between the per cent. total RNase in a solution and the per cent. free RNase, was small and difficult to determine accurately. This also in primarily due to the small molecular weight of RNase, which however on a molar basis is bound to anti-RNase about as effectively as BSA is to anti-BSA, as will be seen below.

Ultracentrifugation.—The ultracentrifuge patterns in this system (Fig. 2) are similar to those obtained with other systems,[3,4,15] taking into ac-

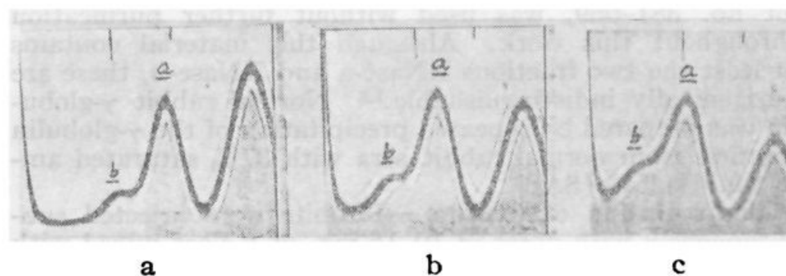

Fig. 2.—Ultracentrifuge patterns of mixtures of RNase and rabbit anti-RNase antibody. Patterns (a), (b) and (c) represent solutions 7, 8 and 9, respectively, of Table II. Sedimentation occurs to the left. The slowest peak in each pattern is due to free RNase and the peaks labelled *a* and *b* to Ag–Ab complexes.

count the differences in molecular weights of the protein antigens. Three major peaks are evident, with sedimentation constants S_w^{20} of about 2.0, 8.0 and 11.0 *S*, corresponding to free RNase, a-complex, and b-complex components,[3,4] respectively. As the Ag excess is decreased, the area under b-complex peak increases relative to that of the a-complex.[12] Thus, as the zone of precipitation is approached, larger aggregates are favored.

The values of the apparent % free RNase in the ultracentrifuge patterns agree well with corresponding values calculated from the independent electrophoresis experiments (Table II). However, the diffusional spreading of the free RNase boundary is so severe that the accuracy of the area measurements is limited. For this reason, no attempt was made to determine the effect of temperature on the relative area under the free RNase peak, and from this a measure of the ΔH for the Ag–Ab reaction as was carried out with the BSA and OA systems.[3,4]

The Equilibrium Constant and Free Energy Change.—The calculations of these quantities were made with the assumptions and by the methods used previously,[3,4] which have been extensively validated.[10] The Ab was taken as completely active and entirely bivalent[3,4]; the assumption was made that all Ag sites were intrinsically equivalent and similarly for all Ab sites; and use

(14) L. G. Longsworth, in "Electrophoresis," ed. by M. Bier, Academic Press, Inc., New York, N. Y., 1959, p. 127.

(15) S. J. Singer, *J. Cell. and Comp. Physiol.*, **50**, Suppl. 1, 51 (1957).

TABLE II

DATA FOR RNASE-ANTIBODY MIXTURES

Soln.[a]	ΔD[b]	N[c]	$N/\Delta D$	Total RNase,[d] %	Free RNase Electroph.,[e] %	Free RNase Ultracentr., %	$K_i \times 10^{-3}$ $f = 2$	$K_i \times 10^{-3}$ $f = 3$	$K_i \times 10^{-3}$ $f = 4$
7	2.921	28.73	9.84	54.0	47.9	49	3.2	2.3	1.7
8	2.943	23.30	7.92	44.1	37.4	35	3.1	2.3	1.6
9	2.903	16.33	5.63	34.2	24.9	25	15	17	13
10	2.694	27.06	10.00	54.9	49.0	..	2.0	2.7	3.7

[a] Solutions 7, 8 and 9 prepared with pure Ab preparation designated A in ref. 9; solution 10 with Ab designated B. [b] Relative refractive increment of solution in arbitrary instrument units. [c] Fractional number of fringes in ascending free RNase boundary. [d] Known from proportions of RNase and Ab solutions mixed. [e] Determined from equation % Free RNase = 5.463 $N/\Delta D$ −5.85, from calibration experiments of Table I.

was made of the Goldberg equation[16,17] for the distribution of Ag–Ab aggregates in such a model system. The intrinsic equilibrium constant, K_i, in l./mole is given by[16,10]

$$K_i = \frac{M_A p}{2C_A(1 - p)\left(1 - p\frac{fC_G M_A}{2C_A M_G}\right)} \quad (1)$$

in which C_A and C_G are the concentrations of total Ab and total Ag, respectively; M_A and M_G are the molecular weights of Ab and Ag, taken for this system as 1.6×10^5 and 1.4×10^4, respectively; f is the Ag valence; and p is the fraction of Ag sites in a solution which have reacted at equilibrium, determined from the relation (2) in which C_{01} is the concentration of free Ag determined electrophoretically and C_G is known.

$$p = 1 - (C_{01}/C_G)^{1/f} \quad (2)$$

The antigen valence, f, for RNase is not unambiguously known at the present time. It is most probably greater than 2, since Ag–Ab precipitate formation is highly unlikely in a system containing a bivalent Ag and a bivalent Ab and which is characterized by the usual value of K_i.[18] The RNase–Ab system, however, exhibits the normal type of precipitation behavior.[7,8] The value f = 4 seems best to fit the experimental data from precipitin titrations.[7] As is shown in Table II, however, the present ambiguity in the value of f introduces an uncertainty in K_i which is no larger than our experimental error.

The calculations are summarized in Table II, with value of K_i given for assumed f values of 2, 3 and 4. The K_i values for solution 9 are much larger than those for solutions 7, 8 and 10, but our experience with other systems and the extra difficulties expected in this system lead us to regard the close agreement of the latter values as fortuitous and to average all four values. Therefore, for an f value of 4, $K_i = (4 \pm 2) \times 10^3$ liters/mole and $\Delta F_i^0 = -4.5 \pm 0.2$ kcal./mole at 1°.

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

(17) S. J. Singer and D. H. Campbell, *ibid.*, **75**, 5577 (1953).

(18) S. I. Epstein, P. Doty and W. C. Boyd, *ibid.*, **78**, 3306 (1956).

Discussion

Before this average ΔF_i^0 value is compared with those we have obtained for other systems, it should be noted that the antibody used in this study was isolated by a procedure which involved exposure to acid conditions.[9] However, with BSA and OA Ab purified by the same method, no material change in ΔF_i^0 was observed, compared to the value obtained with the corresponding Ab not exposed to acid.[9] It is also true that Ab was only about 25% of the amount originally present in the α-globulin fraction and that therefore some fractionation of the Ab might have been introduced by the purification procedure.

With these limitations in mind, it is nevertheless clear that the value $\Delta F_i^0 = -4.5$ kcal./mole for the RNase system is very similar to the corresponding values −4.9 and −5.1 for the BSA and OA systems.[3,4,19] In addition, if synthetic haptens are coupled to proteins and their reaction with specific anti-hapten Ab is investigated, ΔF_i^0 values of the same magnitude are encountered.[10,20] Enough diverse systems involving precipitating rabbit antibodies have now been quantitatively studied to make it highly probable that this close similarity of average ΔF_i^0 values is not accidental but holds generally, independent of the chemical nature of the antigen or antigenic determinant. A ΔF_i^0 value of −5 kcal./mole is quite low and reflects the relatively weak binding which exists between Ag and Ab molecules in aqueous solution. The similarity of ΔF_i^0 for hapten–Ab and protein Ag–Ab interactions suggests that the area of intimate complementary contact between an antigenic site on a protein Ag molecule and its specific Ab is of the same relatively small magnitude as that between a hapten and its Ab. Significantly stronger Ag–Ab interactions, probably involving larger areas of complementary contact, must somehow be selected against, and theories of the influence of Ag on the biosynthesis of antibody must be able to explain this fact satisfactorily.

(19) The K values in refs. 3 and 4 can be converted to K_i values by multiplying by $2/f$, where f = 6 for BSA and 5 for OA.

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