Antibody Affinity. II. Effect of Immunization Interval on Antihapten Antibody in the Rabbit*

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ABSTRACT: The affinity of antihapten antibody was evaluated as a function of the length of the immunization period. Purified antibody in a yield of 75–80% was prepared from rabbit antisera, induced with a large haptenic group (formula wt 540), covering a range of intervals between primary and secondary injections of 2–10 weeks. Association constants were determined by equilibrium dialysis with a tritiated hapten (formula wt 297) allowing concentration measurements to 10^{-8} M. This hapten (amino dye) constituted the terminal portion of the haptenic group. The association constant for this hapten increased over the period studied from 1.2×10^6 to 3.4×10^7 M^{-1} without an apparent plateau. The hapten hippurate, on the other hand, reached a maximum value of 1×10^4 M^{-1} within a 4-week interval. It was estimated that a hapten equivalent to the complete haptenic group would exhibit an affinity after prolonged immunization of at least 10^{10} M^{-1}.

The temperature dependence of the binding of the amino dye showed a large negative enthalpy and a large unfavorable change in entropy. Binding of this hapten by 5- and 3S peptic fragments revealed no energetic role for the C-terminal halves of the heavy chains and no interaction between the two combining regions of the bivalent 7S antibody molecule.

In a previous report (Saha *et al.*, 1966) the preparation of a relatively large haptenic group (SUp),[1] with a formula weight of 540, has been described together with the purification and interaction of the homologous rabbit antibody. This haptenic group was selected, among other reasons, because of its presumed similarity, in size and chemical nature, to the antigenic determinants of proteins. It was anticipated that the antihapten antibody induced by the SUp group would exhibit high affinity for large haptens which constitute a major portion of the SUp group.

In order to induce antibody of maximum affinity in the binding of haptens it is necessary to utilize optimum immunization conditions. It has been clearly demonstrated recently by Eisen and Siskind (1964) that the affinities of anti-2,4-dinitrophenyl antibodies in the rabbit increased progressively with increasing interval between initial injection and time of bleeding. This temporal dependence was most evident with small amounts of antigen, in which case the affinities continued to increase over a period of at least 8 weeks.

In view of the results presented by Eisen and Siskind (1964) a study was undertaken to evaluate the effect of immunization interval on the affinity of anti-SUp antibody. Since association constants much $>10^5$ l./mole were anticipated, the measurement of free hapten concentrations, as required in equilibrium dialysis, could not be achieved by the usual method of absorption spectrophotometry. The alternative method of fluorescence quenching which has been used for high-affinity systems (Eisen and Siskind, 1964) did not appear suitable on the basis of our earlier application of this method to the anti-SUp system (Saha *et al.*, 1966). Since it was our intention to measure hapten concentrations as low as 10^{-8} M or even lower, it appeared that tritiated haptens would best serve our purpose. Most of the binding experiments described below, therefore, have been carried out with a preparation of the tritiated hapten *p*-(*p*-aminobenzeneazo)hippurate (amino dye).

In addition to the role of immunization interval on affinity, the anti-SUp system has also been used to evaluate the possibility of interaction between the combining sites of the bivalent antibody molecule. A possible energetic role for the C-terminal halves of the heavy chains in the specific binding has also been investigated. These evaluations have been made by quantitative comparison of the binding constants for the 5 and 3.5S peptic fragments with that for the untreated antibody from which they were derived.

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[1] Abbreviations used in this work: SUp, $(CH_2)_4NHCOCH(CH_2COO^-)SCH_2CONHC_6H_4N{=}NC_6H_4CONHCH_2COO^-(C_{25}H_{26}N_6O_7S$, formula wt 540); Hy, hemocyanin; AMSA, *S*-acetylmercaptosuccinic anhydride; HSA, human serum albumin; SDeS, sodium decylsulfate; T-E, solvent containing 0.1 M Tris–HCl, pH 7.5, and 0.001 M EDTA; 2T-E, solvent containing 0.2 M Tris–HCl, pH 7.5, and 0.001 M EDTA; iodo compound, $ICH_2CONHC_6H_4N{=}NC_6H_4CONHCH_2COO^-$; MIA dye, $HOCH_2CH_2SCH_2CONHC_6H_4N{=}NC_6H_4CONHCH_2COO^-$.

Materials and Procedures

Preparation of Immunizing Antigen. The protein conjugate used for the induction of antibody formation against the haptenic group was prepared by a two-step process. Hemocyanin (Hy) obtained from the horseshoe crab, *Limulus polyphemus*, was employed as the protein carrier. It was partially purified by removal of other proteins with one-third saturated $(NH_4)_2SO_4$. The preparation of the antigen, SUp-Hy, was done by a procedure similar to that previously described (Saha *et al.*, 1966) for the preparation of the SUp conjugate of bovine γ-globulin. The first step was the thiolation of the protein by the reaction of lysine residues with *S*-acetylmercaptosuccinic anhydride (AMSA) by the method of Klotz and Heiney (1962). This was followed by the reaction of the acquired thiol groups with the sodium salt of *p*-(*p*-iodoacetylaminobenzeneazo)hippuric acid (iodo compound). The synthesis of the iodo compound has previously been described by Saha *et al.* (1966) and AMSA was prepared by the procedure of Klotz and Heiney (1962).

The preparation was carried out with 1 g of Hy in 50 ml of 0.15 M NaCl, 0.02 M phosphate buffer, pH 7.6, under a nitrogen atmosphere. A quantity of 234 mg of AMSA was added and the pH maintained constant with a pH-Stat. After 1 hr at room temperature, 658 mg of the iodo compound was introduced followed immediately by 0.8 ml of 1 M hydroxylamine hydrochloride. Six hours later 3 ml of 0.1 M iodoacetate was added to mask any unreacted thiol groups. After overnight incubation in the cold room, the reaction mixture was clarified by centrifugation and passed through Sephadex G-25 to remove low molecular weight products. The number of SUp groups covalently bound to the protein was estimated after microKjeldahl analysis on the basis of the optical density at 361 mμ and a molar extinction coefficient of 3.0×10^4 for the SUp group (Saha *et al.*, 1966). The batch of SUp-Hy used here contained 12 moles of SUp groups/10^5 g of protein.

Production of Antisera. Antisera were obtained from male New Zealand albino rabbits weighing between 5 and 7 lb. A solution of SUp-Hy (2.5 mg/ml) in 0.15 M NaCl, 0.02 M phosphate buffer, pH 7.4, was emulsified with an equal volume of complete Freund's adjuvant (Difco Co., Cleveland, Ohio). Each rabbit of every group received an initial dose of 2 mg of antigen divided equally among the four footpads. A booster dose of 2 mg was given the same way to all groups except one but the interval between the two doses was varied among the several groups. This interval covered the range from 2 to 10 weeks and each group consisted of eight rabbits. On days 7 and 9 following the booster, 40 ml of blood was taken from each animal by cardiac puncture and on day 11 the rabbit was exsanguinated. Qualitative precipitin tests were positive for every serum and the sera of each group were pooled.

Preparation of Precipitating Antigen. For the specific precipitation of anti-SUp antibody the SUp conjugate of human serum albumin (HSA) was used. The albumin (Cutter Laboratories, Berkeley, Calif.) was reacted by the same two-step process used for Hy and the product (SUp-HSA) contained 17 moles of the SUp group/10^5 g of protein.

Quantitative Precipitin Analysis. Prior to the purification of antibody each pool of antiserum was analyzed for its content of precipitable anti-SUp antibody with SUp-HSA. Samples (1 ml) of antiserum were used and the reaction mixtures were incubated at 37° for 1 hr and at 4° for 2 days. The washed specific precipitates were dissolved in 0.05 ml of 0.2 N NaOH followed by 3 ml of 0.1 M sodium decylsulfate (SDeS), 0.02 M phosphate buffer, pH 7.8. After complete dissolution the optical density was read at 361 and 280 mμ. The antigen and antibody content of the precipitates was calculated using the known extinction coefficients for SUp-HSA at 361 and 280 mμ and the value of 14.4 for $E^{1\%}$ at 280 mμ for the antibody.

Purification of Anti-SUp Antibody. The procedure for the preparation of purified anti-SUp antibody is based on the solubilization of the specific precipitate with the hapten *p*-(*p*-aminobenzeneazo)hippurate (amino dye) and the subsequent removal of the antigen and the hapten by column fractionation. A detailed description of this procedure has been given by Saha *et al.* (1966) and a modified version of it was employed in the present investigation. The anti-SUp antibody was precipitated from the serum pool with the equivalent amount of SUp-HSA and the mixture incubated for 1 hr at 37° and 2 days at 4°. The well-washed precipitate was extracted at 37° with a solution containing 0.2 M amino dye, 0.001 M EDTA, and 0.1 M Tris–HCl buffer, pH 7.5. One milliliter of this solution was used for every 100 mg of precipitated antibody. The extraction procedure was repeated twice and practically all of the antibody was solubilized.

The removal of the antigen and virtually all of the free amino dye was effected by the use of a column of DEAE-cellulose connected in tandem to a column of Sephadex G-25. The first column (i.d. 2.2 cm) contained 1.5 g of DEAE-cellulose (0.9 mequiv/g)/100 mg of antibody and was operated at 37° with 2T-E[1]. The second column (i.d. 2 cm), also prepared with 2T-E, contained 7.5 g of Sephadex G-25 100 mg of antibody and was operated at room temperature. The application of the extract to the first column was preceded by 2 ml of 0.1 M amino dye in T-E[1] and the flow rate was maintained at 5 ml/hr. The extract was followed by the same solution with a volume of 2.5 ml/100 mg of antibody. The columns were developed with 2T-E and 10-ml fractions were collected. The antibody-containing fractions were pooled and the optical density was read at 280 and 390 mμ to allow calculation of the recovery of antibody. The solution was then concentrated to 10 ml by ultrafiltration and lyophilized.

The next stage of purification served to remove the remaining amino dye which was largely bound to the antibody. This was done by displacing this hapten in the presence of a high concentration of a weakly bound hapten, namely, hippurate. Column fractionation was again used to separate both haptens from the antibody

and, combined with dialysis, to obtain thereby a hapten-free solution of antibody. The lyophilized antibody preparation was dissolved in 10 ml of 1 M hippurate adjusted to pH 7.8 and containing 0.001 M EDTA. This solution was placed on a column (2.3 × 25 cm) of Sephadex G-25 prepared with the same solvent. This column was connected to a second column (4 × 35 cm) also containing Sephadex G-25 but prepared in a medium without hippurate, namely, 0.15 M NaCl, 0.002 M phosphate, pH 7.8, and 0.001 M EDTA. The antibody solution was followed by the hippurate-containing solvent at the rate of 5 ml/hr. The first 20 ml of effluent from the first column was discarded and the next 30 ml was allowed to pass into the second column. At this point all of the antibody was contained in the second column. Into this column was then fed the solvent without hippurate at the rate of 18 ml/hr and 5-ml fractions were collected. The protein-containing fractions were pooled and concentrated by ultrafiltration. The antibody solution was finally dialyzed *vs.* a 50-fold greater volume of 0.15 M NaCl, 0.002 M phosphate, pH 7.8 at 4° for 4 days with three changes of the solvent. The antibody preparation was stored in the frozen state.

Preparation of Tritiated Amino Dye. This was prepared from [2-^{3}H]-*p*-aminohippuric acid (New England Nuclear Corp., Boston, Mass.) with a specific activity of 30 mc/mmole. The acid (33 μmoles) was diazotized in a 3-ml volume at 0–5°. To this solution was added 36 μmoles of aniline-ω-methylsulfonate in 0.2 ml of 1 M phosphate buffer, pH 7.4. After overnight incubation of the reaction mixture in the cold room, the amino dye was obtained by hydrolysis for 1.5 hr at 100° following addition of 0.1 ml of 5 N NaOH. The product precipitated at room temperature and was separated by centrifugation. It was purified by solution in 0.5 ml of water and precipitated in 4 M NaCl; three such cycles were carried out. The final yield was 25% of the theoretical value.

The purity of the product was evaluated by comparison with the preparation of the nonradioactive amino dye previously described (Saha *et al.*, 1966). The absorption spectrum of the radioactive product was virtually identical with that of the original synthesis. It showed an absorption maximum at 388 mμ and a minimum at 313 mμ with a ratio of optical densities of 5.26. Chromatographic analysis was carried out by ascending chromatography on Whatman no. 3 paper using an aqueous solvent with 25% dimethylformamide by volume. A sample containing 0.16 μmole of the tritiated amino dye yielded only one colored and one coincident spot of radioactivity when analyzed by a strip-chart scanner. The specific activity of the dye when assayed as described below was 4650 cpm/mμmole.

Preparation of Peptic Fragments. Both 5 and 3.5S peptic fragments of purified anti-SUp antibody were prepared. A separate pool of antiserum obtained as described above with an 8-week interval between the primary and booster injections was used for the purification. The peptic digestion was carried out by published procedures (Nisonoff *et al.*, 1960a) and the separation of the 5S product from the smaller fragments effected by gel filtration through Sephadex G-75 (Utsumi and Karush, 1965). The nitrogen recovery in the 5S component was 56.4%. On the basis of a molecular weight of 150,000 for the untreated antibody and 92,000 for the 5S fragment (Utsumi and Karush, 1965), this recovery corresponds to 92% of the theoretical value.

The conversion of the 5S product to 3.5S fragments followed a method developed by Dr. Utsumi in this laboratory which was based on the procedure used for the preparation of half-molecules (Palmer and Nisonoff, 1964). A solution of 50 mg of 5S protein in 5 ml of 0.014 M mercaptoethanol containing 0.1 M acetate buffer, pH 5.5, and 0.001 M EDTA was incubated at 37° for 24 hr. The thiol groups were then alkylated by the addition of 3 ml of 2 M Tris–HCl buffer, pH 8.5, containing an amount of sodium iodoacetate equivalent to the mercaptoethanol. The reaction was allowed to proceed for 1 hr at room temperature with the pH maintained at 8.3 by the addition of 2 M Tris and then further in the cold room overnight. The 3.5S product was separated from aggregated material and low molecular weight constituents by filtration through a column (3 × 140 cm) of Sephadex G-75. This procedure yielded a recovery of 91% of the initial 5S protein. The value of $E^{1\%}_{1cm}$ at 280 mμ for both the 5 and 3.5S fragments was found to be 15.0. The sedimentation coefficients of the fragments were measured by analytical ultracentrifugation.

Amino Dye Binding by Equilibrium Dialysis. This was carried out with an initial volume of 1 ml in each chamber of the dialysis cell, as previously described (Karush, 1957; Karush and Karush, 1966). The solvent was 0.02 M Tris–HCl buffer, pH 8.5, and 0.15 M NaCl. Triplicate samples for each initial concentration of hapten were used and binding measurements were done at 25.0 and 37.2°. The antibody concentration was usually *ca.* 1 × 10^{-6} M and its exact value was obtained from microKjeldahl analysis of the stock solution based on a molecular weight of 150,000. Because in most experiments the bulk of the hapten was bound to antibody a shorter equilibration time was required if antibody–hapten mixtures were dialyzed *vs.* the solvent then if the reactants were initially located on opposite sides of the dialysis membrane. After equilibration 0.5-ml portions were removed from the protein-free chambers and counted with 10 ml of Bray's solution (Bray, 1960) in a Packard liquid scintillation counter. Generally, counting times were sufficient to yield at least 1000 counts. The casing adsorption correction expressed as a percentage of the total free dye ranged from 9.7 to 17.0% at 25° and was 10% at 37°. Reliable measurement of free hapten concentration could be made down to a value as low as 1 × 10^{-8} M.

Results

Properties of Purified Antibody. Quantitative precipitin analysis was carried out with all of the pools used for antibody purification. The maximum precipitable antibody of six pools together with the recovery of antigen in the precipitate at equivalence is

TABLE I: Antibody Content of Serum Pools of Anti-SUp Antibody.

Pool	Interval between Injections (weeks)	Antibody Protein (mg/ml)	Antigen Recovered (%)[a]
A	2	0.95	65
B	4	0.72	82
C	6	0.56	93
D	8	0.57	91
E	10	0.74	90
F[b]	..	0.44	87

[a] This is per cent of the quantity of added antigen which was recovered in the precipitate at the equivalence point. It was calculated from the optical density at 361 mμ of the dissolved specific precipitate. [b] No booster injection was given to this group of rabbits. They were bled during the 13th week following their single injection.

shown in Table I. Except for the pool which was prepared without a booster injection, the extremes of antibody content fell within a twofold range. The anti-SUp titer was apparently unaffected by the variation in the interval between primary and secondary injections. There was, however, a considerable effect of this interval on the recovery of antigen at equivalence since only after a 6-week delay was the maximum value of *ca.* 90% reached. It should also be added that longer incubation than 2 days at 4° did not alter the quantity of antibody precipitated nor the recovery of antigen at equivalence. Supernatant tests gave negative results for both antigen and antibody at equivalence.

The recovery of purified antibody achieved by the procedure described above represents a substantial improvement over that obtained in the earlier studies with the anti-SUp system (Saha *et al.*, 1966). Over-all recoveries ranged from 75 to 80% of the precipitable antibody in each pool. The bulk of the loss occurred during the first stages of column separation, namely, the removal of the antigen (SUp-HSA) by chromatography with DEAE-cellulose, since the recoveries following this step ranged from 80 to 85%. We may surmise that this loss was due to the retention on the column of soluble antigen–antibody complexes. The use of a dissociating hapten of greater affinity than the amino dye, and of equal solubility, would presumably further improve the yield of antibody. The uniformly high recovery from all of the pools was particularly fortunate for the present study since the comparative properties of the several preparations of purified antibody could then be related to the conditions of their production. In particular the relation sought between affinity and the interval between injections was not rendered obscure by artificial selection during purification.

The absorption spectra of the purified antibody preparations were measured after extensive dialysis *vs.* a solvent containing 0.15 M NaCl and 0.002 M sodium phosphate buffer, pH 7.8. All preparations showed an absorption maximum at 279 mμ and a minimum at 251 mμ with the ratio of optical densities ranging from 2.66 to 2.70. The values of $E^{1\%}_{1cm}$ at 279 mμ based on microKjeldahl analyses and the assumed content of 16% N ranged between 14.3 and 14.6. Thus all of the preparations showed identical spectral properties within experimental error.

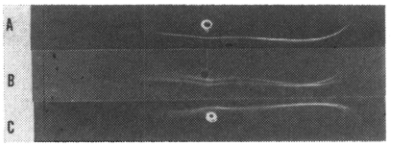

FIGURE 1: Immunoelectrophoresis of purified anti-SUp antibody. (A) Sheep antiserum to rabbit serum. (B) Goat antiserum *vs.* the heavy chain of rabbit γG-globulin. The antiserum was absorbed with the 5S peptic fragment and the Pep-III′ fragment (Utsumi and Karush, 1965). (C) Goat antiserum to the light chains of rabbit γG-globulin.

The presence of contaminating antigen or hapten was evaluated quantitatively by preparing 4% solutions of the antibody and measuring the optical density at 361 mμ for antigen and at 390 mμ for the amino dye. The observed values of optical density demonstrated that the contamination with SUp-HSA did not exceed 0.2% and that there was <0.04 mole of amino dye/mole of antibody in every preparation.

All of the antibody preparations were examined in the analytical ultracentrifuge (Spinco Model E) at 59,780 rpm in a solvent containing 0.01 M sodium phosphate buffer, pH 7.8, and 0.2 M NaCl. Each one gave a single symmetrical peak throughout the run and they were all of the 7S variety.

Immunoelectrophoresis of Purified Antibody. In view of the antigenic multiplicity of the equine antihapten antibodies previously found (Rockey *et al.*, 1964), the anti-SUp preparations were examined by immunoelectrophoresis. This was first carried out with sheep antiserum to rabbit serum, generously provided by Dr. Sheldon Dray, the antiserum containing antibodies to the various immunoglobulins of rabbit serum. A single long precipitin line typical of γG-immunoglobulin was observed with each preparation (Figure 1A).

It has been found previously that rabbit γG-globulin consists of two antigenic types associated with its heavy chains (Utsumi and Karush, 1965). Further antigenic analysis of the anti-SUp antibody was therefore carried out with goat antiserum to the heavy chains of rabbit γG-globulin. The serum was absorbed with the 5S peptic fragment and the Pep-III′ fragment (Utsumi and Karush, 1965) since such absorption facilitated the appearance of a double line. In the present case double precipitin lines were also observed (Figure 1B) which were similar to the γGa and γGb components of equine antihapten antibody (Klinman *et al.*, 1966). This be-

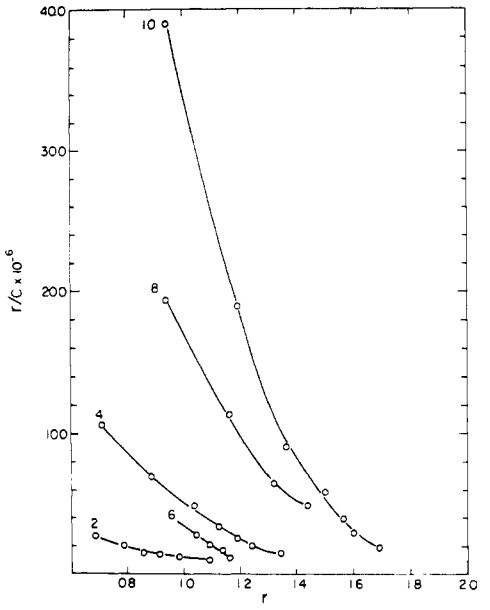

FIGURE 2: The effect of immunization interval on the binding at 25° of amino dye by purified anti-SUp antibody. The interval in weeks between the primary and secondary injections is indicated for each binding curve.

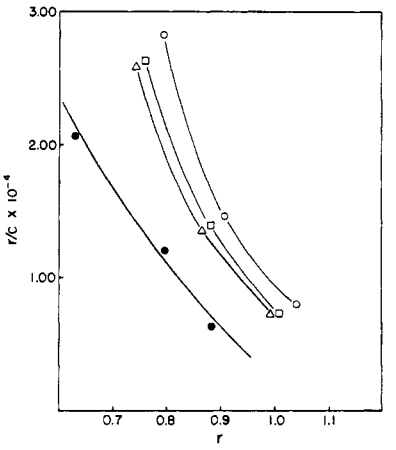

FIGURE 3: The effect of immunization interval on the binding at 25° of hippurate by purified anti-SUp antibody. The interval in weeks between primary and secondary injections was as follows: ●, 2; ○, 4; □, 6; Δ, 10.

havior was found with all of the preparations and served to substantiate the original conclusion (S. Utsumi, unpublished data) that there is antigenic heterogeneity in that portion of the heavy chain which corresponds to peptic fragment Pep-V (Utsumi and Karush, 1965).

The antibody preparations were also tested by immunoelectrophoresis with goat antiserum prepared against the isolated light chains of rabbit γG-globulin (Utsumi and Karush, 1964). Double lines were again observed for all of the preparations (Figure 2C) as was found with the equine γG antibodies (Klinman *et al.*, 1966).

Chromatographic Analysis of Antibody. Further characterization of the anti-SUp preparations as well as additional evidence of their similarity was obtained by chromatography with DEAE-cellulose. A column (1.5 × 35 cm) was prepared with this adsorbent (1 mequiv/g) and equilibrated with 0.02 M sodium phosphate buffer, pH 8.0. A portion of each anti-SUp preparation was dialyzed *vs.* this buffer in the cold room for 20 hr. The slight amounts of precipitate were removed by centrifugation and a sample of 2.5 ml (20 mg of protein/ml) was applied to the column. Chromatography was done at room temperature and a total volume of 420 ml was collected in 6-ml fractions with the starting buffer. The recovery of protein before the gradient was applied ranged from 78 to 80% for all of the preparations and no significant difference among the shapes of the elution curves was noted. The subsequent gradient, ranging from the initial buffer to 0.30 M sodium phosphate buffer, pH 8.0, served to elute the remaining 20% of protein. No striking variation was observed in this portion of the chromatogram either.

A further examination of the antibody eluted by the gradient was made by immunoelectrophoresis. The appropriate fractions were pooled and the protein concentrated by ultrafiltration. Each concentrate was used at 10 mg/ml with sheep antiserum to rabbit serum. The precipitin bands were found to coincide with at least a portion of the γG band. It was concluded, therefore, that all of the anti-SUp preparations were γG-immunoglobulins.

Binding of Amino Dye. The binding data were plotted in the form of r/c *vs.* r (Scatchard, 1949; Karush and Karush, 1966), where r is the average number of hapten molecules bound per antibody molecule at the free equilibrium hapten concentration c. This procedure was followed to allow the accurate evaluation of the average association constant (K_0) which was taken as the reciprocal of the free dye concentration corresponding to half-saturation of the antibody, *i.e.*, $r = 1$. This value of the association constant corresponds to the maximum value of the Gaussian and Sips distribution functions when the free energy of complex formation (or ln K) is the independent variable in these functions (Karush, 1962). The nonlinearity of the plots of r/c *vs.* r serves to reveal the heterogeneity of the antibody populations with respect to their affinity for the amino dye.

The initial concentrations of the hapten in the equilibrium dialysis experiments were selected to encompass a range of r, for every pool, which would include the value of $r = 1$. This result was achieved with all of the preparations except one (pool C) in which the lowest experimental value of r was 1.04. In this case a short but entirely reliable extrapolation was necessary for the evaluation of K_0. The total range of r covered in the experiments was from 0.6 to 1.7 and a composite plot

TABLE II: Binding of Amino Dye and Hippurate by Purified Preparations of Anti-SUp Antibody at 25°.

		Amino Dye				Hippurate	
Prepn	Interval (weeks)	K_0 (l./mole $\times 10^{-6}$)	$-\Delta F_u$ (kcal/mole)	Heterogeneity Index[a] a	σ	K_0 (l./mole $\times 10^{-4}$)	$-\Delta F_u$ (kcal/mole)
A	2	1.23	10.7	0.60	2.6	<0.5	<7.4
B	4	5.15	11.5	0.50	3.5		
C	6	3.40	11.3	...	...	1	7.8
D	8	17.3	12.2	...	...		
E	10	34.3	12.7	0.50	3.5		
F[b]	..	13.0	12.1	...	...	...	...

[a] The indices a and σ are associated with the Sips and Gauss distribution functions, respectively (Karush, 1962). The value of σ was obtained from a using the equation given in the Appendix. [b] No booster injection was given to this group of rabbits. They were bled during the 13th week following their single injection.

of the binding results is shown in Figure 2. The average association constants obtained from these curves are listed in Table II together with the unitary free energies calculated from them (Karush, 1962). It is evident that there is a continuous increase in affinity with increasing interval between the two injections. Within the extremes of this interval, namely, 2 and 10 weeks, there was almost a 30-fold increase in K_0 (1.23×10^6 to 3.43×10^7 M^{-1}).

The heterogeneity of binding is represented by the indices a and σ in Table II. The index σ is a measure of the dispersion of the Gauss distribution function (in ΔF) which is assumed to describe the heterogeneity (Karush, 1962). The index a is associated with the closely related Sips distribution function (Sips, 1948). This function leads to a binding equation which is a generalized form of the adsorption isotherm as follows

$$r/n = \frac{(K_0 c)^a}{1 + (K_0 c)^a}$$

in which n is the valence of antibody. The values of K_0 and a in Table II were obtained by plotting log $[r/(n - r)]$ *vs.* log c. The values of σ were calculated from the equation relating a and σ (see Appendix).

Binding of Hippurate. In view of the strong binding of the amino dye with the later sera, it appeared feasible to study the binding of the hippurate ion. A knowledge of its affinity was of interest since the hippurate molecule constituted a portion of the amino dye and such information would facilitate the evaluation of the energetic contribution of different portions of the hapten to the affinity.

The binding studies were carried out with [1-^{14}C]-hippurate (6 mc/mmole, New England Nuclear Corp.) by equilibrium dialysis at an antibody concentration of 3.00×10^{-5} M and 25° in the same solvent used for the amino dye. The method for the radioassay of free hippurate was the same as that employed for the amino dye. The range of values of r covered experimentally

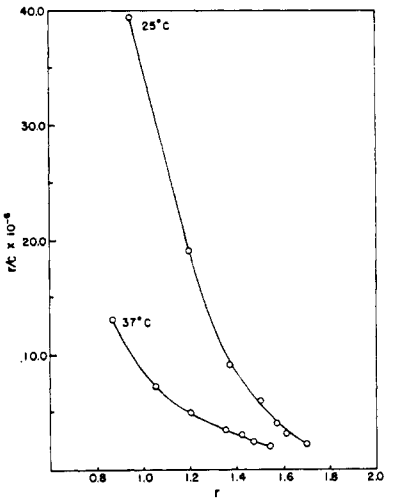

FIGURE 4: The temperature dependence of the binding of amino dye by anti-SUp antibody purified from 10-week antiserum.

was from 0.6 to 1.05 corresponding to a free hapten concentration range from 2.80×10^{-5} to 1.40×10^{-4} M. The binding curves obtained with four preparations of anti-SUp antibody are shown in Figure 3 and the average affinities are listed in Table II. It is clear that K_0 for hippurate reaches the limiting value of 1×10^4 M^{-1} within the 4-week interval. The implication of this observation is that this value represents energetically the maximum extent of interaction between the anti-SUp antibody and hippurate.

Temperature Dependence of Binding. The temperature dependence of the binding of amino dye by 10-week antibody (pool E) was evaluated to obtain a thermodynamic characterization of the interaction. This was done several weeks after the results found with pool E and shown in Table II were obtained. A common series of antibody–dye mixtures were used in the protein side

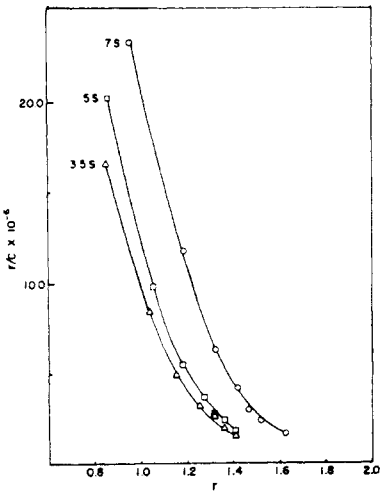

FIGURE 5: The binding of amino dye by 8-week anti-SUp antibody and by the 5 and 3.5S peptic fragments derived from it.

of the dialysis cell for the measurements at 25.0 and 37.2°. A considerable decrease of affinity was found at the higher temperature as may be seen in Figure 4. The values of K_0 were 3.4×10^7 M^{-1} at 25.0° and 8.4×10^6 M^{-1} at 37.2°. The former value is in agreement with the figure of 3.4×10^7 M^{-1} for the same pool (Table II). The thermodynamic calculations were carried out in the usual way yielding a value for ΔH of −21.6 kcal/mole of hapten. At 25° the unitary free energy (ΔF_u) was −12.65 kcal/mole and the unitary entropy change (ΔS_u) was −30.1 eu/mole.

Binding by Fragments of Antibody. Measurements were made of the binding of the amino dye by antibody purified from a separate 8-week pool and by the 5 and 3.5S peptic fragments derived from this antibody. The results of these binding experiments are given in Figure 5. The calculation of the values of r for the fragments was based on a molecular weight of 92,000 for the 5S fragment and 2 × 46,000 for the 3.5 fragment (Utsumi and Karush, 1965). The association constants and the corresponding unitary free energies for the three preparations are listed in Table III together with the values of the heterogeneity index a. This index was calculated as described above.

TABLE III: Binding of Amino Dye by Anti-SUp Antibody and Fragments.

Prepn	K_0 (l./mole × 10^{-7})	$-\Delta F_u$ (kcal/mole)	a^a
7 S	2.09	12.4	0.42
5 S	1.25	12.1	0.42
3.5 S	1.02	11.9	0.43

[a] This is the heterogeneity index of the Sips distribution function.

Discussion

The analysis of the affinity of purified antibody from sequential sera demonstrates quite clearly an increase in the average association constant for the binding of the amino dye (Table II). The increase in the interval between the initial and secondary injections from 2 to 10 weeks gave rise to a 30-fold increase in this constant. The increased affinity was apparently associated with an increased heterogeneity. This may be inferred from the greater value of a at 2 weeks (0.6) compared to that at 10 weeks (0.5). These results are in agreement in both respects with the observations of Eisen and Siskind (1964) on rabbit antidinitrophenyllysyl antibodies. This concordance with diverse kinds of haptenic groups serves to substantiate the notion that the observed temporal dependence of affinity is a general and intrinsic aspect of the immune response.

The 30-fold range revealed in the present investigation probably does not represent the maximum range of average affinities of pooled antisera for the binding of the amino dye. In the earlier investigation with anti-SUp antibody (Saha *et al.*, 1966) the average association constant for the binding of the amino dye was 1.08 × 10^5 l./mole. Although this value was obtained with a 3-week interval between the primary and secondary doses, the 2-week value obtained in the present study is more than 10-fold larger. The explanation for this apparent paradox is not clear but the use of higher doses of antigen (5 *vs.* 2 mg) in the earlier study and the administration of the booster dose intravenously rather than subcutaneously in emulsion form may constitute the main factors involved. In any case the 300-fold range represented in the two studies is a minimum measure of the development of affinity since a plateau value was not encountered in the time range studied here.

The binding of hippurate exhibited a different time dependence from that of amino dye. As is apparent from Table II within an interval of 4 weeks the anti-SUp antibody had achieved its maximum affinity for hippurate, the terminal portion of the SUp haptenic group. Energetically this maximum corresponds to a unitary free energy of 7.8 kcal/mole of hapten.

The maximum observed contribution of the *p*-aminophenylazo group to the binding of the amino dye can be obtained from the unitary free energies in Table II by difference. The value of 4.9 kcal/mole (12.7–7.8) for this group may be compared to the value previously obtained (Karush, 1956) of 4.1 kcal/mole for the phenyl group in another system. These results suggest that the value of 4 kcal/mole may generally represent a close approximation of the energetic contribution of the phenyl group to the affinity of the antigen–antibody complex. In any case the value of 4.9 kcal may not represent the full energetic capacity of the *p*-aminophenylazo group.

The unitary free energy of 12.7 kcal does not, in all likelihood, represent the maximum affinity which the

anti-SUp antibody might exhibit. Aside from a greater affinity for the amino dye itself, which antibody beyond the 10-week interval may possess, the potential area of specific interaction probably extends beyond that portion of the SUp group corresponding to the amino dye. Substantial evidence for this is provided by the results of the earlier study with the anti-SUp system (Saha *et al.*, 1966). In this study it appeared that MIA dye, prepared by the reaction of β-mercaptoethanol with the iodo compound, was bound to the anti-SUp antibody with an average association constant 200-fold greater than that for the amino dye. It is reasonable to anticipate therefore that the SUp group itself or the equivalent hapten would be bound to 10-week antibody with an average association constant as least as large as 10^{10} l./mole.

The temperature dependence of the binding of amino dye by 10-week antibody is quite striking (Figure 4). From the limited results available it appears that the association reaction is due to a large decrease of enthalpy (ΔH = 21.6 kcal/mole of hapten) and that the entropy change is large and unfavorable (ΔS_u = −30.1 eu/mole). This result is not in accord with thermodynamic expectations if hydrophobic interaction were the main source of stability (Karush, 1962) and its interpretation remains obscure. It is noteworthy that the energetic results found here accord very closely with those for the antidinitrophenyllysyl system with rabbit antibody (Eisen and Siskind, 1964).

A comparison of the binding properties of the peptic fragments with that of the 7S antibody is shown in Table III. The difference of only 0.3 kcal for the binding of the amino dye between the 7 and 5S molecules indicates that the C-terminal halves of the heavy chains are not essential for the maintenance of the complementary structure of the antibody combining sites nor are they involved in any energetically significant interaction with the hapten. The further fragmentation of the 5S molecule into the monovalent 3.5S fragments leads again to no significant alteration of the binding behavior. From the constancy of the free energies and, particularly, from the virtual identity of the values of a, it may be concluded that there is no interaction between the two combining regions of the 7S antibody. Thus, the specific interaction of one region with a ligand does not influence the thermodynamic reactivity of the other site. At this level of analysis, therefore, there is no evidence of allosteric transformation associated with antibody activity. These results confirm the earlier conclusions reached by Nisonoff *et al.* (1960b) in their study of the papain fragments of purified antihapten antibody and are in accord with a tripartite structure of the γG molecule in which each portion is structurally autonomous (Nisonoff *et al.*, 1960b; Noelken *et al.*, 1965).

Appendix

An analytic relation between the heterogeneity indices σ of the Gaussian distribution function (w) and a of the Sips function (N) may be readily derived. If the functions are expressed in terms of the free energy they take the following normalized forms

$$w(\Delta F) = (1/\sigma\sqrt{\pi}) \exp[-(\Delta F_0 - \Delta F)^2/(RT\sigma)^2]$$

$$N(\Delta F) = (1/\pi) \times \frac{(\sin \pi a) \exp[(a/RT)(\Delta F_0 - \Delta F)]}{1 + 2 \cos \pi a \exp[(a/RT)(\Delta F_0 - \Delta F)] + \exp[(2a/RT)(\Delta F_0 - \Delta F)]}$$

where ΔF_0 corresponds to the maximum value of the distribution functions. If we set the condition that $w(\Delta F_0) = N(\Delta F_0)$ then

$$1/\sigma\sqrt{\pi} = (0.5\pi)[\sin \pi a/(1 + \cos \pi a)]$$

from which it follows that

$$\sigma = 2\sqrt{\pi}[(1 + \cos \pi a)/(\sin \pi a)]$$

This may be written in the alternative form

$$\sigma = 2\sqrt{\pi}(\cot \pi a/2)$$

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