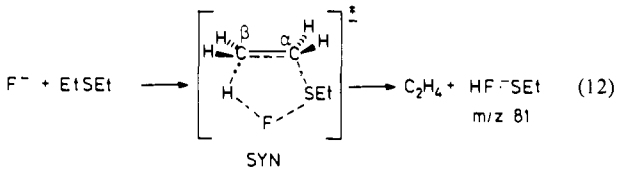
the C_β–H–F bond angle must be larger than in the discussed E2C model. On the other hand, the extraordinarily large leaving group effect of 1.23 suggests that the leaving group in the transition state is involved in both bond breaking and bond formation, which points to interaction of the leaving group and the base in the transition state. In the previous paper similar isotope and leaving group effects were obtained for the elimination reaction of diethyl ether induced by OH^- and producing water solvated ethoxide anions.[10] It was argued that this reaction could best be described by a syn elimination with a cyclic transition-state structure. Following the same argumentation the formation of the HF solvated thiolate anions is also considered to proceed via a syn elimination mechanism (eq 12). This mechanism strongly favors solvated thiolate



(12)

anion formation because of the interaction of F^- and the leaving thiolate group in the transition state as a consequence of which no reorganization of the reaction complex is required in order to stabilize $HF\cdot^-SEt$. This is in contrast with the E2H and E2C mechanisms where solvation of this thiolate anion can only be achieved after a considerable reorganization of the reaction complex.

Conclusions

It appears that the results of the present study confirm our conclusion from the previous study,[10] that the concept of the variable transition state structure is applicable to base-induced elimination reactions in the gas phase. Perturbations in transition-state character effected by changing the leaving group or the base seem to follow the rules established for condensed phase β-elimination reactions.[33] That is, from both the present and the previous study it follows that increasing the base strength not only enhances the carbanionic character of the transition state but also causes the transition state to shift toward the reactant side of the reaction coordinate. Both effects also find expression if the leaving ability of the leaving group is improved, as follows from comparison of the mechanisms of the reactions of OH^- with diethyl ether and diethyl sulfide. A more reactantlike transition state is to be expected upon substitution of the ethoxy by an ethyl thiolate group. However, the enhancement of the carbanionic character is more difficult to rationalize, unless it is assumed that development of negative charge on the β-carbon is better stabilized by sulfur than by oxygen.

Another interesting conclusion which follows from combination of the results of the present study with those of the previous study[10] is that, as the overall exothermicity of the elimination reactions becomes very small, the E2H mechanism, characterized by a linear proton transfer, is superseded by a mechanism, which is characterized by a bent proton transfer and a two-side attack of the base on the substrate. This is illustrated by the reactions of OH^- with diethyl ether and F^- with diethyl sulfide. For both reaction systems the free ethoxide and free ethyl thiolate anion formation, respectively, are close to thermoneutral.[16,21] Evidently, as a result of this, the E2H mechanism in the reaction of OH^- with diethyl ether has to compete with a syn elimination mechanism in which a bent proton transfer and interaction of the incoming base with the leaving group in the transition state promotes the formation of water solvated ethoxide anions. Moreover, the E2H mechanism in the reaction of F^- with diethyl sulfide is completely superseded by elimination mechanisms involving a bent proton transfer. These are the E2C mechanisms, characterized by a two-side attack of F^- on the β-hydrogen and the α-carbon and a syn elimination mechanism characterized by a two-side attack on the β-hydrogen and the leaving group, analogous to the syn elimination mechanism which is operative in the OH^-/diethyl ether reaction system.

In summary, the exothermicity of the elimination reactions appears to be a selection criterion for the elimination mechanisms, where the entropically favored linear proton transfer mechanism (E2H) is preferred for relatively highly exothermic elimination reactions, whereas the enthalpy favored bent proton transfer mechanisms with rigid transition states (E2C and syn) are preferred in the case of slightly endothermic elimination reactions.

Acknowledgment. We thank Mr. F. A. Pinkse for his expert technical assistance and the Netherlands Organization for Pure Research (SON/ZWO) for financial support.

Registry No. $S(C_2H_5)_2$, 352-93-2; D_2, 7782-39-0.

(33) Thornton, E. R. *J. Am. Chem. Soc.* **1967**, *89*, 2915.

Direct Examination of Chemical Kinetic Laws by Visual Imagery. 3.† Association of Latex Particles Modified with Antigens and Antibodies

Hiromi Kitano,* Satoshi Iwai, Tsuneo Okubo, and Norio Ise

Contribution from the Department of Polymer Chemistry, Kyoto University, Kyoto, Japan.
Received April 27, 1987

Abstract: Association processes of latex particles modified with antigen (human serum albumin, HSA) and antibody (anti-HSA immunoglobulin G) are examined by direct visual observation and by a spectrophotometric method. By direct visual observation using an ultramicroscope connected to an image-processing system, the rate constant of the dimeric association process of polymer latex particles carrying 16000 antigens or antibodies is estimated to be 1.3×10^7 M^{-1} s^{-1} at 25 °C, which is smaller than that of the association reaction of oppositely charged polymer latex particles (1.9×10^9 M^{-1} s^{-1}), but is larger than the rate constant of the association reaction between an antigen and an antibody (1.9×10^6 M^{-1} s^{-1}). The activation parameters of the interlatex reaction system are also estimated.

Latex particles have been extensively used in clinical assays as carriers of antigens or antibodies to detect complementary antibodies or antigens in biological samples by the agglutination method.[1] Many reports have been published on practical conditions of clinical assays using polymer latices. This agglutination phenomenon of latex particles, however, has not yet been clarified

† Part 1: see ref 3a; part 2, see ref 3b.

(1) (a) Rembaum, A.; Yen, S. P.; Cheong, E.; Wallace, S.; Molday, R. S.; Gordon, I. L.; Dreyer, W. J. *Macromolecules* **1976**, *9*, 328. (b) Crane, L. J. *Clin. Chem.* **1981**, *27*, 697. (c) Bernard, A. M.; Moreau, D.; Lauwerys, R. R. *Ibid.* **1982**, *28*, 1167.

0002-7863/87/1509-7608$01.50/0

completely. For example, von Schulthess et al. extensively examined the size distribution of aggregates of human serum albumin (HSA) coated latices by anti-HSA antibody using light scattering and resistive pulse counting technique.[2] However, they did not focus on the kinetic problem in latex–latex association.

The initial stage of the agglutination reaction involves binary association of latex particles. We report here a kinetic study of the dimeric association of latex particles which carry an antihuman serum albumin–immunoglobulin G (anti-HSA-IgG) and a human serum albumin (HSA) on their surfaces. The association reaction was followed by a direct visual observation method using an ultramicroscope connected to an image-processing system and by a spectrophotometric technique.

In previous papers we reported on the direct visual observation of a dimeric association process of oppositely charged polymer latex particles by taking advantage of latex particles being large enough to be seen with an ultramicroscope.[3a,b] We could estimate directly, or "with the naked eye", the rate constant of the binary association of latex particles. To our knowledge, such a direct visual observation of the chemical reaction in solution has not been reported.

Experimental Section

Materials. Human serum albumin (HSA) was purchased from Sigma, St. Louis, Mo. Antiserum (from rabbits) which contained anti-HSA–immunoglobulin G (anti-HSA-IgG) was purchased from MBL Co., Nagoya, Japan; the anti-HSA-IgG was purified at 4 °C by conventional procedures,[4] namely, precipitation by 40% saturated ammonium sulfate, fractionation with a DEAE-cellulose column using 0.01 M phosphate buffer (pH 8.0), and immunological chromatography with a HSA-containing Sepharose 4B column (1.6 cm × 50 cm) (180 mg of HSA was bound onto 100 mL of Sepharose 4B gel using a BrCN activation method).[5] Glycine–HCl buffer (pH 2.3) was used as an eluting buffer. Elution of the purified anti-HSA-IgG from the column was monitored at 280 nm using a mini-UV monitor (Type II, Atto Co., Tokyo, Japan). After dialyses with H_2O and phosphate buffer (pH 7.8), the antibody solution was condensed using an ultrafiltration apparatus. The immunological activity of the antibody was checked by the Ouchterlony method using a double immunodiffusion disc from Miles Co. after each purification step.

Acrolein from Tokyo Kasei Co., Tokyo, Japan, was distilled before use. Styrene from Wako Pure Chemicals, Osaka, Japan, was washed twice with 1 N NaOH and once with water and afterwards dried over anhydrous magnesium sulfate. *N*-(2-Hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES) was obtained from Dojin Chemical Laboratories, Kumamoto, Japan. Other reagents were commercially available. Milli-Q grade water was used for preparation of solutions.

Latex Particles. Two kinds of acrolein-containing latex particles (AL-1 and AL-2) were prepared by the emulsifier-free polymerization. In the case of AL-1, acrolein (10 mL, 0.15 mol) and styrene (17.5 mL, 0.15 mol) were copolymerized in 300 mL of water at 60 °C under continuous stirring with a Teflon paddle at 300 rpm. Nitrogen gas was continuously passed through the flask. Potassium peroxydisulfate (250 mg) dissolved in 5 mL of H_2O was added as an initiator. For AL-2, 17.5 mL of styrene and 10 mL of acrolein were copolymerized in 250 mL of H_2O at 60 °C under continuous stirring at 280 rpm. The acrolein-containing latex particles obtained were purified by washing with distilled water using an Amicon ultrafiltration apparatus with a Millipore membrane (VMWP14200, pore size 0.05 μm). The ultrafiltration process was repeated until the absorbance of the filtrate at 254 nm became negligible. The latex particles were finally suspended in water (39 mg of solid/mL of H_2O for AL-1 and 15.6 mg/mL for AL-2).

The diameters of the latex particles were estimated to be 2100 Å (AL-1) and 3750 Å (AL-2) from electron micrographs using a JEM-100U electron microscope (Nihon Denshi Co., Tokyo, Japan). From elemental analyses the contents of acrolein residues in the latex particles were estimated to be 32 and 19% for AL-1 and AL-2, respectively.

Preparation of Antigen- and Antibody-Carrying Latex Particles. HSA (90 mg) was dissolved in 27 mL of water, in which 10 mL of the AL-1 latex suspension was added. The pH of the suspension was adjusted to 9.0 using 0.1 N NaOH and HCl solution and the mixture was continuously stirred overnight at 4 °C. The suspension mixture was poured into an Amicon ultrafiltration apparatus and the absorbance of the filtrate at 280 nm was checked to determine the amount of protein bound to the latex surface. During the ultrafiltration 1.5 mL of ethanolamine was added to block unmodified aldehyde groups in the latex. The HSA-carrying latex obtained was mixed with 2.5 g of dimethylamine–borane complex to moderately reduce a Schiff base formed between the latex and the protein.[6] The suspension mixture was ultrafiltrated again, and finally the HSA-carrying latex was suspended in H_2O (HSA-AL-1, 11.3 mg/mL). A similar procedure was used for the preparation of an anti-HSA-IgG-carrying latex (anti-HSA-IgG-AL-1, 14.9 mg/mL).

To increase the immunological sensitivity of the protein-carrying latex, a spacer group was introduced between the AL-1 latex and the protein. An excess amount of 6-aminohexanoic acid was coupled with the acrolein latex overnight at pH 9.0 and room temperature. Then the Schiff base formed was reduced using a dimethylamine–borane complex. The spacer-carrying latex particle obtained (C_6-AL-1) was purified by the ultrafiltration and coupled with HSA at pH 4.5 using a water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC).[12] The HSA-carrying latex was purified by ultrafiltration and finally suspended in H_2O (HSA-C_6-AL-1, 16.4 mg/mL). A similar procedure was used for the preparation of anti-HSA-IgG-carrying latex (anti-HSA-IgG-C_6-AL-1, 10.1 mg/mL).

In the case of AL-2, a similar procedure was used for the preparation of an antigen- or an antibody-carrying latex with a spacer (HSA-C_6-AL-2 (60 mg of protein/g of latex) and anti-HSA-IgG-C_6-AL-2 (143 mg/g of latex)). By changing the amount of the antibody or the antigen in the reacting suspension, we could obtain the latex particles with various amounts of antibodies or antigens on their surfaces.

We also prepared a latex particle carrying aminohexyl side chains by the reaction of AL-2 (234 mg) with an excess amount of hexamethylenediamine (25 g) at pH 9.0. The aminohexyl-AL-2 latex (A-C_6-AL-2) was purified by repeated washing with water using the ultrafiltration apparatus. The antibody was bound to the aminohexyl-AL-2 by the EDC method (anti-HSA-IgG-A-C_6-AL-2, 52 mg of protein/g of latex).

Infrared Measurements. An infrared spectrum of the latex was observed to detect carbonyl groups in the latex using an infrared spectrophotometer (IR-440, Shimadzu, Kyoto, Japan).

Microscope Measurements. A direct observation of the association reaction of the latex particles was carried out using an ultramicroscope (AXIOMAT, Carl-Zeiss) connected to an image-processing system (IBAS, Carl-Zeiss). Association processes were recorded on a video tape, and, by a slow replay of the video tape, the percents of monomeric particles in a visual field were estimated at appropriate intervals. The observation cell was thermostated using a Neslab RTE-8 water bath.

Kinetic Measurements. The association process of the protein-carrying latex particles initiated by the addition of a protein which is complementary to the protein bound to the latex particle was observed spectrophotometrically at 680 nm using a high-sensitivity spectrophotometer (SM 401, Union Engineering, Osaka, Japan). Within 5 s after the mixing of the reacting suspension using a polyethylene rod, a spectroscopic observation was started and continued for 100–200 s. The association process of the antigen–latex with the antibody–latex was also examined in a similar manner. The association reaction of the anti-HSA-IgG with HSA in the homogeneous system was followed using a conductance stopped-flow (CSF) apparatus.[7,8] The observation cell of these apparatuses was thermostated using a Lauda water bath.

Determination of ζ Potential of Latex Particles. ζ potentials of the polymer latex particles were measured using a laser ζ-potential meter (Laser Zee™ Model 501, Pen Kem Inc., Bedford Hills, NY).

Results and Discussion

A. Modification of Latex Particles with Proteins. The absorbance of filtrates (280 nm) from the ultrafiltration apparatus was checked in the purification step of latex particles, and it was estimated that 1.3×10^{-15} g of anti-HSA-IgG and 4.8×10^{-16} g of HSA were bound onto the AL-1 latex particle (anti-HSA-IgG-AL-1, HSA-AL-1), respectively. Theoretical amounts of protein bound onto one AL-1 latex particle in a so-called "end-on"

(2) von Schulthess, G. K.; Benedek, G. B.; De Blois, R. W. *Macromolecules* **1980**, *13*, 939.
(3) (a) Kitano, H.; Iwai, S.; Ise, N. *J. Am. Chem. Soc.* **1987**, *109*, 1867. (b) Kitano, H.; Iwai, S.; Ise, N.; Okubo, T. *Ibid.*, in press.
(4) Williams, C. A., Chase, M. W., Eds. *Methods in Immunology and Immunochemistry*; Academic Press: New York, 1967; Vol. I.
(5) (a) Cuatrecasas, P.; Anfinsen, C. B. *Methods Enzymol.* **1971**, *22*, 345. (b) Cuatrecasas, P. *J. Biol. Chem.* **1970**, *245*, 3059.
(6) Geoghegan, K.; Cabacungan, J. C.; Dixon, H. B. F.; Feeney, R. E. *Int. J. Peptide Protein Res.* **1981**, *17*, 345.
(7) Okubo, T.; Ishiwatari, T.; Kitano, H.; Ise, N. *Proc. R. Soc. London, Ser. A* **1979**, *366*, 81.
(8) Okubo, T.; Hongyo, K.; Enokida, A. *J. Chem. Soc., Faraday Trans. 1* **1983**, *80*, 2087.

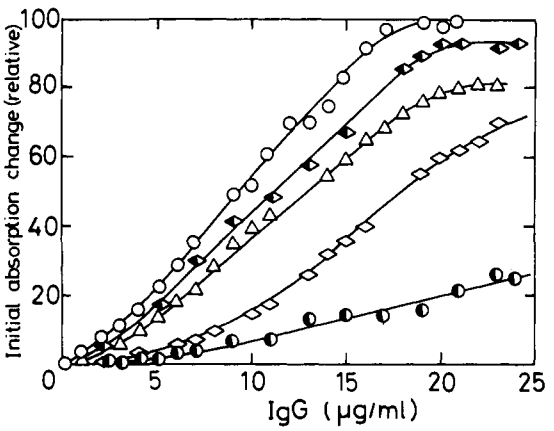


Figure 1. Influence of the antibody concentration on the initial absorption change at various pH at 25 °C, [HSA-C_6-AL-1] = 5.3 × 10^{-11} M: ◑, pH 6.0, ◇, pH 7.0, △, 8.0, ○, pH 9.2, ◆, pH 10.2.

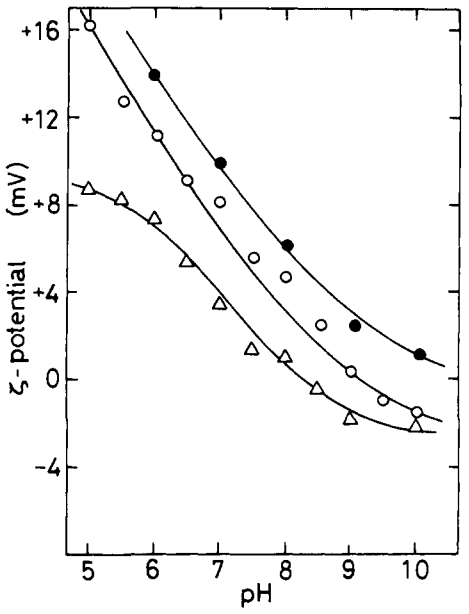


Figure 2. The pH dependence of the ζ potential of the latex particles modified with proteins: ●, anti-HSA-IgG-A-C_6-AL-2; ○, anti-HSA-IgG-C_6-AL-2; △, HSA-C_6-AL-2.

manner were estimated to be 2.3 × 10^{-15} g for IgG and 1.1 × 10^{-15} g for HSA using the theoretical occupation areas of the protein molecules (1256 Å^2 for HSA and 1520 Å^2 for IgG).[9] By the introduction of spacer, the amount of bound protein greatly increased to 6.4 × 10^{-15} g of IgG and 5.2 × 10^{-15} g of HSA per particle (anti-HSA-IgG-C_6-AL-1, HSA-C_6-AL-1), which are a little larger than the theoretical values calculated for a single layer of proteins. This increase was partly due to the decrease in steric hindrance for the approach of protein to the carboxyl group on the latex surface, and mainly due to the formation of several layers of proteins coupled by EDC on the latex surface.

B. Immunological Reaction of Modified Latex-Free Protein System. After rapid mixing of a small amount of HSA solution with the anti-HSA-IgG-AL-1 suspension in a quartz observation cell using a small polyethylene rod, the absorbance of the suspension at 680 nm increased rapidly. Figure 1 shows the effect of added HSA on the initial absorption changes of the HSA-C_6-AL-1 suspension at various pHs. The initial absorption change almost linearly increased at first and leveled off with further increase in the HSA concentration, probably because of the steric hindrance at a high concentration of anti-HSA-IgG; the anti-HSA-IgG already bound on the HSA-AL-1 sterically hinders the

(9) (a) Fair, B. D.; Jamieson, A. M. *J. Colloid Interface Sci.* **1980**, *77*, 525. (b) Uzgiris, E. E.; Fromageot, H. P. M. *Biopolymers* **1976**, *15*, 257.

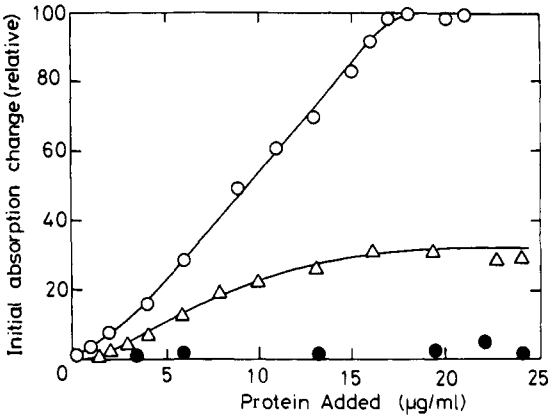


Figure 3. Effect of the protein concentration on the initial absorption change at pH 9.2 and 25 °C: ○, HSA-C_6-AL-1 + anti-HSA-IgG; △, HSA-AL-1 + anti-HSA-IgG; ●, HSA-C_6-AL-1 + human IgG; [antigen-latex] = 5.3 × 10^{-11} M.

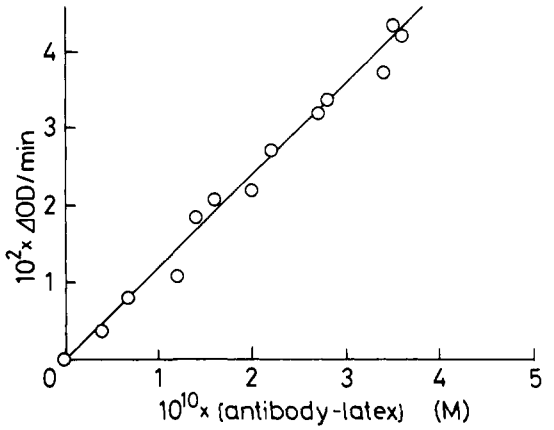


Figure 4. Influence of the concentration of the antibody–latex on the initial absorption change at 25 °C, pH 8.7.

diffusion of newly approaching anti-HSA-IgG to the immobilized HSA which still remains free. A similar tendency was observed in the anti-HSA-IgG-carrying latex-free HSA system.

Figure 1 also shows the effect of pH on the initial absorption change. It is clear that at pH 9 the latex with a spacer shows the largest sensitivity to the added complementary protein. Figure 2 shows the effect of pH on the ζ potentials of the antigen- and the antibody-carrying latex particles. In the pH region where ζ potentials of the particles are small, the sensitivity of the immunological reaction was the highest according to Figure 1. The modified latex particles HSA-C_6-AL-2 and anti-HSA-IgG-C_6-AL-2 showed approximately 0 V at pH 8.2 and 9.2, respectively, which reflects the influence of the isoelectric points (pI) of the introduced proteins to the ζ potentials of the modified latex particles (pI of HSA, 4.7–5.2, and that of IgG, 5.8–7.3[10]).

Figure 3 shows the effect of a spacer on the sensitivity of the HSA-carrying latex to the added anti-HSA-IgG. By the introduction of the spacer the immunological sensitivity increased markedly, partly because of the decrease of steric hindrance[11] and also because of the decrease of disadvantageous conformational changes of the bound protein caused by the direct immobilization onto the latex surface.[12,13] It should be noted here that the nonspecific aggregation was not observed; filled circles in the figure show the additional effect of an ordinary (nonspecific) human IgG to the HSA-carrying latex suspension.

By the conventional agglutination measurements on microtiter plates for 6 h, 40 ng mL^{-1} of IgG and 20 ng mL^{-1} of HSA were

(10) Putman, F. W., Ed. *The Plasma Proteins*, 2nd ed.; Academic Press: New York, 1975-1977; Vol. I–III.
(11) Goldstein, L. *Methods Enzymol.* **1976**, *44*, 435.
(12) Kitano, H.; Nakamura, K.; Ise, N. *J. Appl. Biochem.* **1982**, *4*, 34.
(13) Kitano, H.; Ise, N. *Biotechnol. Bioeng.*, in press.

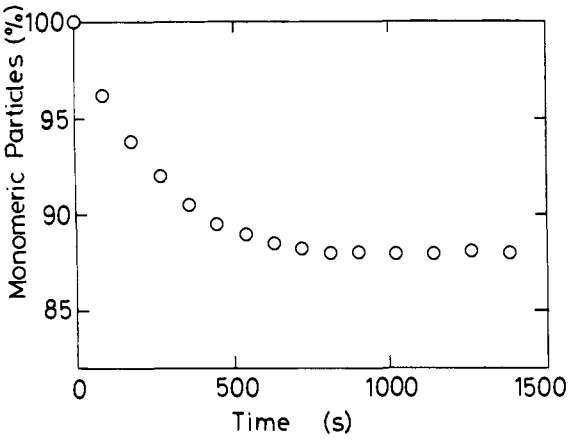


Figure 5. Time dependence of the percent of monomeric latex particles at 25 °C, pH 8.7: [anti-HSA-IgG-C_6-AL-2] = 3.6×10^{-10} M, [HSA-C_6-AL-2] = 4.0×10^{-11} M.

detectable with the naked eye using spacer-containing latices. The minimum concentration of detectable HSA (20 ng mL^{-1} = 300 pM) was satisfactorily small enough for practical diagnostic purposes.

C. **Immunological Reaction between Latex Particles Modified with Complementary Proteins. a. Correlation between Spectroscopic and Microscopic Methods.** The interparticle reaction between the anti-HSA-IgG-C_6-latex and the HSA-C_6-latex was examined by both turbidity measurement and direct visual observation of latex particles using an ultramicroscope. From the turbidity measurements with [anti-HSA-IgG-C_6-AL-2] $\gg$ [HSA-C_6-AL-2], we could observe a linear relationship between ΔOD min^{-1} and [anti-HSA-IgG-C_6-AL-2] added (Figure 4), where 1 M of the HSA-C_6-AL-2 suspension denotes 6.02×10^{23} particles of the HSA-C_6-AL-2 in 1 L of the suspension. Using an image-processing system connected to an ultramicroscope[16] we could obtain the time dependence of the percents of monomeric latex particles in the visual field. At time = 0, only monomeric particles of the anti-HSA-IgG-C_6-AL-2 were found to be present in the visual field, and by the addition of a small amount of HSA-C_6-AL-2 suspension into the observation cell using a polyethylene mixing rod, the number of monomeric particles decreased with time and almost level off (Figure 5). Though not shown in the figure, the number of dimeric particles in the visual field was observed to increase with time corresponding to the decrease in the number of monomeric particles. Long after the start of the association (>15 min), further association processes of dimeric particles with other particles to form trimers, tetramers, and so on were confirmed by microscopic observation. Therefore, the initial turbidity change observed here could be attributed to the binary association of latex particles.

By plotting log [dimer] vs. time by the microscopic measurements, we could estimate the relaxation time (τ) for the association of latex particles. From the slope of the plots of τ^{-1} vs. [anti-HSA-IgG-C_6-AL-2] (Figure 6) we could obtain the second-order rate constant, k_f, for the association of latex particles modified with antigens and antibodies by the equation:

$$\tau^{-1} = k_f[\text{antibody-latex}] + k_b \qquad (1)$$

The k_f value obtained is 1.3×10^7 M^{-1} s^{-1} at pH 9.2 and 25 °C, and it is much smaller than the value obtained for the dimeric association of oppositely charged latex particles (1.9×10^9 M^{-1} s^{-1}),[3] which approximately agrees with the theoretical rate constant for the diffusion-controlled binary association of latex particles of equal size (7.7×10^9 M^{-1} s^{-1}).[20] Since the particles undergo Brownian motion, such a small association rate constant observed here might be partly due to the failure of effective binary collision to form immuno complexes between antigens and antibodies bound onto latex particles as described in section b below. The effective binary collision would result in a dimer formation.

The rate constant of the forward reaction obtained here is a little larger than the value observed for the association of the

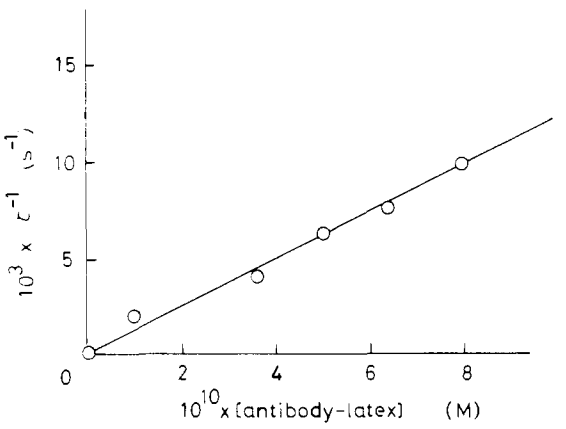


Figure 6. Influence of the concentration of the antibody-latex on τ^{-1} at 25 °C, pH 8.7.

anti-HSA-IgG and the HSA detected by the CSF technique (k_2 = 1.9×10^6 M^{-1} s^{-1} at pH 8.0 and 25 °C), because the local concentration of the reactants is much higher for the latex-bound case than for the free systems. The experimental value for the free HSA-anti-HSA-IgG system is in good agreement with the values reported for other immunological systems (1.0×10^6 M^{-1} s^{-1} (25 °C) and 1.0×10^6 M^{-1} s^{-1} (20 °C) for ovalbuminin-anti-ovalbumin[14] and cytochrome *c*-anti-cytochrome *c* systems,[15] respectively).

b. Effect of Number of Proteins Bound to Latices. On the anti-HSA-IgG-C_6-AL-2 latex, 1.6×10^4 antibodies were attached. To facilitate comparison, the forward rate constant reduced for one antibody molecule ($k_{f,cor}$ = k_f/number of antibodies on the latex surface) was estimated and found to be 810 M^{-1} s^{-1}, which is much smaller than the observed reaction rate constant for the antigen-antibody reaction in the homogeneous system. Wolff et al. reported that 1.3 molecules of IgG per vesicle is enough for interaction of the vesicle with antigen-carrying cells.[17]

With the decrease in the number of antibodies bound to one latex particle, $k_{f,cor}$ increased (k_f = 1.1×10^7 M^{-1} s^{-1} and $k_{f,cor}$ = 1.6×10^3 M^{-1} s^{-1} for the latex to which 7000 antibodies are bound), probably because a too densely packed protein is disadvantageous for the efficient immunological reaction. The corrected k_f value is, however, still much smaller than that for the free system.

In the case of the anti-HSA-IgG-A-C_6-AL-2 (5800 antibodies were bound) which was prepared from the aminohexyl group containing latex particle, both k_f (8.9×10^7 M^{-1} s^{-1}), and $k_{f,cor}$ (1.5×10^4 M^{-1} s^{-1}) values were larger than those for the IgG-C_6-AL-2 system, probably because of the advantageous orientation of the antibodies introduced, and partly because of the electrostatic attraction between the slightly anionic C_6-AL-2 latex and the slightly cationic A-C_6-AL-2 latex. However, the reduced k_f value was still much smaller than that for the free antigen–free antibody system.

Previously we reported that trypsin (M_w 23 000) immobilized onto the polymer latex was inhibited only by 30% of its activity by the addition of an excess amount of free soy bean trypsin inhibitor (STI; M_w 21 700)[18], which suggests that the binding site of the antigen and the antibody bound onto the latex surface do not always direct outwards from the latex surface (advantageously for the association reaction). This orientation factor largely diminishes the concentration of effective antigens and antibodies on the latex surface. Such a disadvantageous steric factor induces a failure of a dimer formation of the modified latex particles which

(14) Levison, S. A.; Jancsi, A. N.; Dandliker, W. B. *Biochem. Biophys. Res. Commun.* **1968**, *33*, 942.
(15) Noble, R. W.; Reichlin, M.; Gibson, Q. H. *J. Biol. Chem.* **1969**, *244*, 2403.
(16) Ito, K.; Nakamura, H.; Ise, N. *J. Chem. Phys.* **1986**, *85*, 6136; **1986**, *85*, 6143.
(17) Wolff, B.; Gregoriadis, G. *Biochim. Biophys. Acta* **1984**, *802*, 259.
(18) Kitano, H.; Nakamura, K.; Ise, N. *J. Appl. Biochem.* **1982**, *4*, 487.

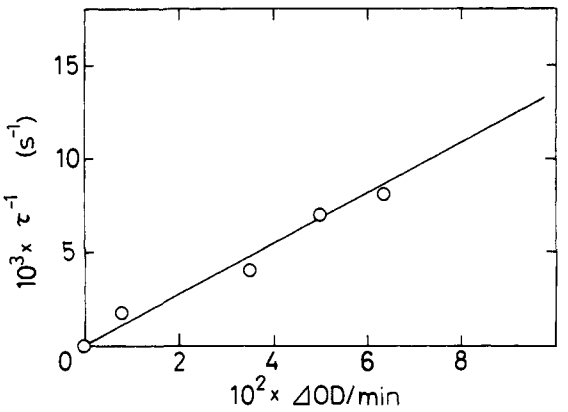


Figure 7. Plots of τ^{-1} vs. initial absorption change at 25 °C, pH 8.7.

Table I. Activation Parameters for the Dimeric Association of HSA-C_6-AL-2 and Anti-HSA-IgG-C_6-AL-2 Latex Particles

$\Delta G^{\ddagger}$	(kcal mol^{-1})	7.7 ± 0.1	(4.8,[a] 3.9[b])
$\Delta H^{\ddagger}$	(kcal mol^{-1})	9.3 ± 0.1	(7.4,[a] 4.4[b])
$\Delta S^{\ddagger}$	(eu)	5 ± 2	(8.8,[a] 1.5[b])

[a] Association between oppositely charged latices.[13] [b] Theoretical values for diffusion-controlled association.[13]

are undergoing Brownian motion.

In the case of the HSA-anti-HSA-IgG reaction, the reactants are much larger than trypsin and STI (M_w of HSA, 66 000, and that of IgG, 155 000). Therefore, the orientation factor described above would be much more important in the HSA-anti-HSA-IgG system. In addition, the restriction of conformational changes of the immobilized proteins[12,13] for their associations might also make the k_f value smaller. Because of these factors, the immobilization of the antibody reduces the binding ability of the antibody with the free antigen to one-fifth to one-tenth of that in the free system.[19] Furthermore, the antibodies and the antigens examined here are bound onto the latex surfaces, which reduces the binding ability of the antibody to the antigen markedly. Assuming that the percent of the effective antigen reactive to the antibody is only 1%, $k_{f,cor}$ for the HSA-C_6-AL-2 and anti-HSA-IgG-A-C_6-AL-2 reaction systems would become 1.5×10^6 M^{-1} s^{-1}, which is comparable to the rate constant estimated in the homogeneous system.

c. Activation Parameters for the Association Process. To clarify why the k_f values of the latex system are smaller than those in the homogeneous system, we examined the temperature effect on the interlatex reaction. Figure 7 shows the linear relationship between the reciprocal of the relaxation time (τ^{-1}) and the initial absorption change, which suggests the possibility estimating the reaction rate constant from both the initial absorption change and the direct visual observation. Figure 8 shows the initial absorption change at various temperatures. From the Arrhenius plots of the slopes in Figure 8, we could determine activation parameters $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ for the binary association process (Table I). In parentheses both the theoretical values for the diffusion-controlled binary association of the carrier latex particles and the experimental values for the association of oppositely charged latex particles are shown. The tendencies of the activation parameters of the interlatex reaction examined here are slightly different from those of the association of oppositely charged polymer latex particles (both $\Delta G^{\ddagger}$ and $\Delta H^{\ddagger}$ of the protein-carrying latex system are larger than those of the oppositely charged latex system). The rate constant and the values of the activation energies for the association of the modified latex particles are much different from those calculated for the diffusion-controlled association of latex particles, which suggests that there must be a rate-determining factor which needs a higher activation energy.

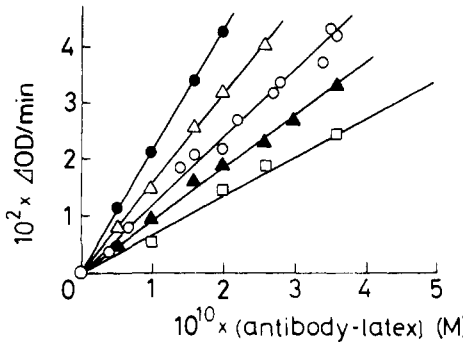


Figure 8. Plots of the initial absorption change vs. concentration of the antibody latex at various temperatures, pH 8.7: □, 15 °C; ▲, 20 °C; ○, 25 °C; △, 30 °C; ●, 35 °C.

The activation parameters ($\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$) of the ovalbumin–anti-ovalbumin were reported to be 12.0 kcal mol^{-1} and 9.1 eu.[14] Thus the activation parameters obtained here seem to partly reflect those of the antigen–antibody reaction. The following factors causing large activation energy may be pointed out: (1) the conformational changes of the charged side chains near the immobilized proteins on the latex surface, and (2) the conformational changes of the proteins themselves accompanying the association of the latex particles.

Acknowledgment. We thank Dr. Hirokazu Hasegawa of our department for taking the electron micrographs of latex particles. This work is supported by a grant-in-aid for the special project research "Design of Multiphase Biomedical Materials" by the Ministry of Education, Science and Culture.

(19) Nakamura, H.; Sugiura, T. In *Methods in Immunological Biochemistry*; Osawa, T., Nagai, K., Eds., Tokyo Kagaku Dojin: Tokyo; 1986; pp 126–129.

(20) (a) Bird, R. B.; Stewart, W. B.; Lightfoot, E. N. *Transport Phenomena*; Wiley: New York, 1960; p 780. (b) Einstein, A. *Ann. Phys.* **1905**, *17*, 549.