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Soluble oligovalent antigen–antibody complexes

II. THE EFFECT OF VARIOUS SELECTIVE FORCES UPON RELATIVE STABILITY OF ISOLATED COMPLEXES

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Summary. Soluble oligovalent antigen–antibody complexes were isolated and analysed by ultracentrifugation to assess the effect of several forces upon the composition and stability of soluble complexes. Complexes were prepared with fluorescein (F) conjugates of rabbit serum albumin (RSA) or thyroglobulin (RTg) and high affinity rabbit anti-F antibodies. Isolated complexes containing two antigen molecules (Ag_2 complexes) tended to dissociate and form an equilibrium with complexes containing one antigen molecule (Ag_1 complexes). This equilibrium was thermolabile, concentration dependent and affected by the original combining ratio and the area in the gradient from which complexes were harvested. Small amounts of free antibody dissociated from soluble complexes also to form a dynamic equilibrium; this equilibrium was much less affected by the above parameters. The data support the concept that complexes grow in size by a process analogous to polymerization of simple subunits and that the driving forces for polymerization are of a lower order of magnitude and more affected by physical variables than the primary reaction between antibody and its antigen.

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INTRODUCTION

A large body of literature is available concerning both theoretical and experimental aspects of the antigen–antibody relationship (recent reviews: Day, 1972; Eisen, 1974; Metzger, 1974; Butler & Beiser, 1974). Most of the early work concerned antigen–antibody precipitates. This was due in part to the inherent difficulty of measuring a soluble reaction product in the presence of free reactants. Moreover, conveniently purified antigens, such as native proteins, possess multiple antigenic determinants, and thus lattice formation and precipitation usually result when such antigens are reacted with antisera. A favoured approach to avoid both of these problems has been to react anti-hapten antibodies with either free hapten or with oligovalent hapten-conjugates. Using these systems in combination with such techniques as fluorescence polarization Dandliker & de Saussure, 1970; Dandliker & Levinson, 1968) and the temperature-jump relaxation method (Froese, Sehon & Eigen, 1962; Froese, 1968), the kinetics of both the association and dissociation reactions have been measured. The experimental evidence is in good agreement that the association reaction rates are uniformly rapid,

while the dissociation reactions are generally much slower and vary over a wide range. Thus, the relative stability of the antigen-antibody bond is mainly determined by the dissociation reaction (Day, 1972; Steward & Petty, 1972; Talmage, 1960).

Dandliker *et al.*, using anti-fluorescein antibodies, found a two-log difference for equilibrium constants obtained using free fluorescein compared with those obtained with flurescein or albumin conjugates (Dandliker, Schapiro, Meduski, Alonso, Feigen & Hamrick, 1964). This difference was explained by the previously described concept of monogamous bivalency (Eisen & Karush, 1949), that is, the thermodynamically favourable condition which occurs when bivalent antibody is sterically able to attach both of its binding sites to the same antigen molecule. The formation of immune precipitates and the solubilization of precipitates in antigen excess has received much attention (De Lisi, 1974; Arend, Teller & Mannik, 1972); less work has been done concerning the formation of simpler, soluble complexes containing small numbers of antigen molecules.

The first paper in this series (van Es, Knutson, Kayser & Glassock, 1979) has examined the effect of the combining ratio, antigen valence and concentration upon the size and composition of soluble complexes. The present study concerns the stability of isolated soluble immune complexes. Complexes were formed from oligovalent fluorescein (F) conjugates of rabbit serum albumin (RSA) or thyroglobulin (RTg) and high affinity anti-F antibodies. Isolated complexes were allowed to come into new equilibria and then analysed in secondary ultracentrifuge runs. The equilibria were considered to be between complexes and free antibody and between larger and smaller complexes. Using derivations from the Law of Mass Action, the equilibrium constants (K_1 and K_2) were estimated, and the effects of several physical parameters upon the constants were analysed.

MATERIALS AND METHODS

The materials and methods for formation of complexes were exactly the same as described in van Es *et al.*, (1979). In this study 3F-RSA and 3F-RTg were used as antigens. Only 10–40% w/w gradients were used for both preparative and secondary ultracentrifuge runs.

Assessment of stability of isolated immune complexes

Complexes were pooled from fractions 8–10 of each gradient and in selected experiments also from fractions 3–5 and fractions 11–13. Portions of each pool were then dialysed against BBS to remove the sucrose and were incubated at 4 or 37° for 24 or 72 h, as appropriate for the experiment. After these incubations, 300 μl samples containing 0·38 to 7·9 pmol of antigen were re-applied to a second gradient which was then ultracentrifuged, fractionated, counted and plotted in an identical manner as in the primary run.

Calculation of equilibrium constants

It was observed that isolated complexes containing two antigen molecules (Ag_2 complexes) tended either to remain intact or dissociate into simpler complexes containing a single antigen molecule (Ag_1 complexes). Conversely, isolated Ag_1 complexes either remained as Ag_1 complexes or self-associated to form Ag_2 complexes. In both cases, negligible Ag_3 complexes were formed at the concentrations studied and small amounts of free antibody were generated. Two equilibrium constants (K_1 and K_2) were therefore derived from the Law of Mass Action for the special circumstances of these experiments.

K_1 represents an approximate measure of the extrinsic equilibrium constant for free antibody (generated by dissociation from isolated soluble complexes) and the available antigen, and it was derived by the following line of reasoning. Assuming that antibodies in soluble hapten-carrier anti-hapten complexes dissociate and associate independently of each other, the general expression for the equilibrium between isolated soluble complexes and the free antibody they generate can be written,

$$Ag_yAb_{xy} \underset{k_a}{\overset{k_d}{\rightleftarrows}} Ag_yAb_{xy-1} + Ab \underset{k_a}{\overset{k_d}{\rightleftarrows}} Ag_yAb_{xy-2}$$

$$+ 2Ab \ldots \underset{k_a}{\overset{k_d}{\rightleftarrows}} Ag_yAb_{xy-n} + nAb,$$

where y is the number of antigen molecules per complex and x is the molar antibody–antigen ratio. In all of our experiments, the quantity of free antibody in equilibrium with isolated soluble complexes was small relative to antibody remaining bound in complexes, indicating that the equilibrium is far to the left. Therefore,

$$K_1 = \frac{k_d}{k_a} = \frac{(Ag_yAb_{xy})}{(Ag_yAb_{xy-1})\,(Ab)}.$$

Since free antibody can be generated from either Ag_2 or Ag_1 complexes,

$$K_1 = \frac{(Ag_2Ab_{2x} + AgAb_x)}{(Ag_2Ab_{2x-1} + AgAb_{x-1})\,(Ab)}.$$

Since for each antibody released there will be either one Ag_2Ab_{2x-1} or one $AgAb_x$ generated, the expression simplifies to:

$$K_1 = \frac{(Ag_2Ab_{2x} + AgAb_x)}{\left(\frac{Ab}{x}\right)^2}$$

In this equation, the free antibody expression is divided by x so that K_1 can be compared for complexes containing widely differing antibody–antigen ratios, i.e. antibodies per antigen molecule. In practice, K_1 was calculated by

$$K_1 = \frac{\text{moles Ag/2 in Fx 7,8,9,10/2} + \text{moles Ag in Fx 10/2,11,12,13} - \text{moles Ab/}x\text{ in Fx 21,22,23}}{(1/\text{vol applied})\left(\frac{\text{moles Ab/}x}{\text{in Fx 21,22,23}}\right)^2}$$

Since fraction 10 clearly contained complexes from both peaks, half of the complexes in fraction 10 were assigned to Ag_1 and half to Ag_2 complexes. Subtracting free antibody in the numerator provided a small correction necessary to convert total complexes present into undissociated complexes. Here, again, Ab was divided by x so as to express equilibrium per antibody and thus facilitate comparisons between preparations of complexes.

K_2 represents the equilibrium constant between complexes and thus measures the tendency of Ag_2 complexes to dissociate into simpler Ag_1 complexes. Thus,

$$Ag_2Ab_{2x} = (AgAb_x)_2 \underset{k_a}{\overset{k_d}{\rightleftarrows}} 2AgAb_x$$

$$K_2 = \frac{(Ag_2Ab_{2x})}{(AgAb_x)^2}$$

In practice, K_2 was calculated by

$$K_2 = \frac{\text{moles Ag/2 in Fx 7,8,9,10/2}}{(\text{moles Ag in Fx 10/2,11,12,13})^2} \times \text{vol applied.}$$

Complexes in fraction 10 were again arbitrarily divided equally between Ag_1 and Ag_2 complexes.

The percentage of total antigen applied to each gradient recovered in fractions 7–13 was 75·6 ± 0·88 SEM (Range, 67–82%). One gradient had only 36% of ^{125}I activity recovered in these fractions, and the data from this single gradient were excluded.

RESULTS

General

Representative sucrose gradient profiles are shown in Fig. 1 for a primary ultracentifuge run to isolate 3F-RTg soluble complexes (Fig. 1a) and a secondary run to analyse isolated complexes (Fig. 1b). IgG normally peaks in fraction 22, while 3F-RTg (broken line) peaks in fraction 13. Soluble complexes were demonstrated in both gradients by the presence of both antigen and antibody in lower-numbered fractions than when centrifuged alone. The Ab/Ag ratio of complexes in Fig. 1a was about 2·7 and quite constant for complexes of widely differing size. Peaks of complexes were formed in fractions 8–9 and 11–12 in both gradients. The predominant molecular formulae were Ag_2Ab_{5-6} and $Ag_1\,Ab_{2-3}$ for these two peaks in both gradients and were determined as described in van Es *et al.*, (1979). In Fig. 1b, isolated complexes which were originally from fractions 8–10 were seen to have mainly remained Ag_2 complexes. Some complexes dissociated into simpler Ag_1 complexes, however, and a small amount of free antibody was generated as evidenced by the antibody peak in fraction 22. The Ab/Ag ratio in the secondary run was quite similar, comparing Ag_2 and Ag_1 complexes, and was only slightly lower than the ratio of the original Ag_2 complexes (fractions 8–10 in Fig. 1a).

Effect of time of incubation before isolation

Antigen–antibody reactions occur quite rapidly; precipitation, in contrast, may not be complete for hours or even days. Immune complexes incubated for different periods before isolation were therefore studied to see whether equilibrium was reached within 1h of combining antigen and antibody and, if so, to see whether after equilibrium rearrangements within complexes might lead to more stable configurations. Immune complexes were prepared at 10 × antigen excess, equimolarity and 10 × antibody excess with ^{125}I-3F-RSA and ^{131}I-IgG anti-F. After incubation for 1 h at 25°, half of each mixture was ultracentrifuged and the remaining halves incubated an additional 24 h at 4° before they, too, were ultracentrifuged. These primary runs are compared in the top three panels of Fig. 2 by

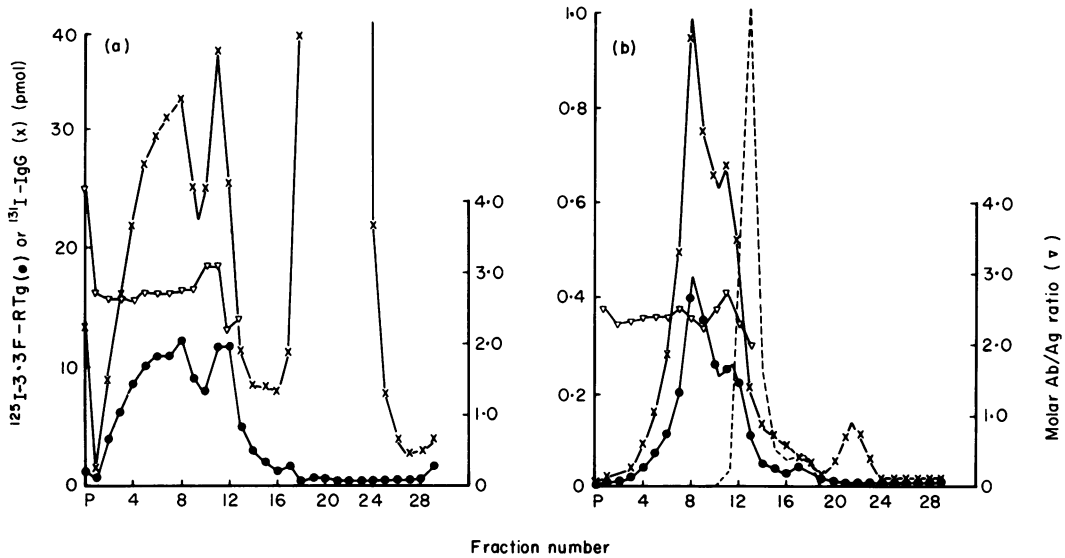

Figure 1. Sucrose gradient profiles for (a) a primary ultracentrifuge run to isolate soluble immune complexes and (b) a secondary run to analyse complexes isolated from fractions 8–10 in the preparative run. ^{131}I-antibody IgG (x) and ^{125}I-3F-RTg (●) are plotted as picomoles per fraction along with the molar Ab/Ag ratio (▽). The normal position of uncombined antigen is shown by the broken line. Ultracentrifugation was in a 10–40% w/w sucrose gradient at 269,000 *g* and 4° for 17 h.

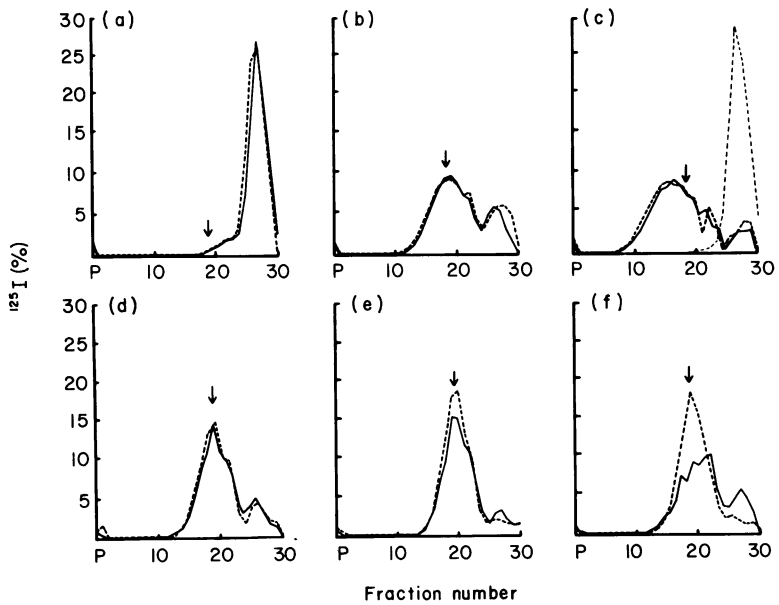

Figure 2. Sucrose gradient profiles of 3F-RSA for primary ultracentrifuge runs (top three panels) and secondary runs (bottom panels). Complexes were prepared in 10 × antigen excess (left), molar equivalence (middle) and in 10 × antibody excess (right) keeping antibody concentration constant. Complexes were incubated for 1h (solid lines) or 24 h (broken lines). Re-runs are depicted directly under the primary run from which they were harvested. Arrows indicate the fractions from which complexes were harvested in the primary runs. The normal position of uncombined antigen (3F-RSA) is shown in (c) by a broken line. Incubations after isolation were for 24 h at 4°. Ultracentrifuge conditions were as in Fig. 1.

plotting percentage ^{125}I versus fraction number. The lines for 1 h and 24 h incubations are nearly superimposable for each top panel, indicating that equilibrium is achieved within 1 h. Fractions 18–20 were harvested from each preparative gradient, dialysed for 24 h at 4°, and re-ultracentrifuged. The bottom three panels in Fig. 2 show the re-runs of complexes isolated from the gradient directly above. Profiles were quite similar for each pair, suggesting that incubation for 24 h before separation did not result in more stable complexes. Because RSA has a molecular weight of only 69,000, discrete peaks of complexes containing various numbers of antigen molecules were not resolved in these gradients. There was, however, a tendency for complexes to dissociate into simpler complexes and free antigen, as shown by the formation of a shoulder of complexes and a small, free antigen peak on the reruns.

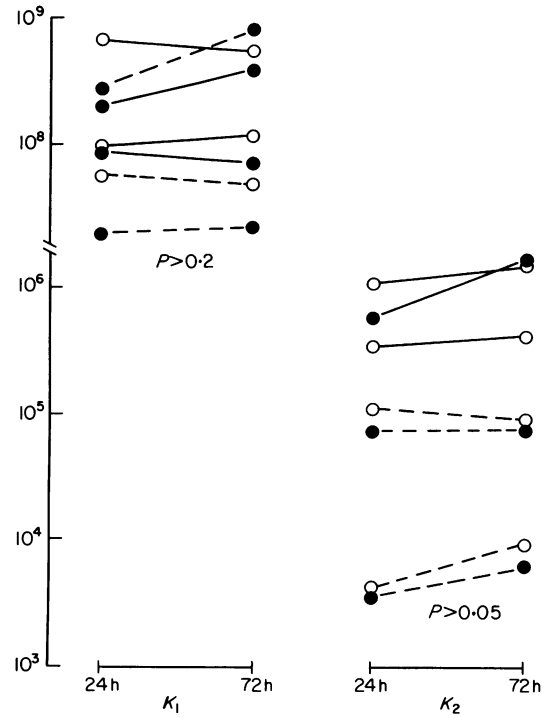

Figure 3. Equilibrium constants for complexes incubated 24 or 72 h after isolation. Complexes were harvested from fractions 8–10 (——)and 11–13 (– – – –) and were incubated at 4 (○) or 37° (●). Values for K_1 and K_2 were calculated as described in the Materials and Methods Section. The P values were obtained from Student's two-tailed t test for paired data.

Effect of time of incubation after isolation

Equilibrium constants K_1 and K_2 were compared for isolated 3F-RTg complexes incubated for 24 or 72 h after isolation in order to determine whether equilibrium was achieved after 24 h of incubation. Complexes were isolated from fractions 8–10 and 11–13 and were incubated at 4 or 37°. The data obtained are shown in Fig. 3. There was no statistical difference detected for either K_1 or K_2 ($P>0{\cdot}2$) comparing incubation times of 24 or 72 h. Therefore, isolated soluble immune complexes in this system achieve apparent equilibrium within 24 h at either 4 or 37°.

In this and in all subsequent experiments, K_1 is seen to be several orders of magnitude higher than K_2. That is, the equilibrium formed between isolated soluble immune complexes and free antibody largely favours the binding of the antibody as measured by K_1. By comparison, Ag_2 complexes have a greater tendency to dissociate (into Ag_1 complexes) as measured by K_2.

Effect of temperature of incubation

The effect of varying temperature of incubation upon the stability of isolated soluble immune complexes was determined by comparing the equilibrium constants K_1 and K_2 obtained after incubation of 3F-RTg complexes at 4° with those obtained after 37° incubation. Immune complexes were prepared at various combining ratios and isolated from ultracentrifuge fractions 8–10. Incubations were for 24 h. A plot of the data is shown in Fig. 4. Both K_1 and K_2 were significantly ($P<0{\cdot}05$) higher than when measured at 37°. The proportional increase was greater for K_2 than for K_1. Thus, the binding forces between complexes and between antibody and complexes were both thermolabile with the forces between complexes being proportionately more thermolabile.

Effect of antibody–antigen combining ratio

The effect of the original antibody–antigen combining ratio upon the equilibrium constants K_1 and K_2 was assessed by comparing K_1 and K_2 for complexes formed in 2–10× antigen excess and in 2–8× antibody excess. Isolated complexes were incubated at 4 or 37°. The pooled data are shown in Table 1. Similar P values were obtained when data from the two temperatures were analysed separately. K_2 is higher for complexes formed in antibody excess ($P<0{\cdot}01$), but

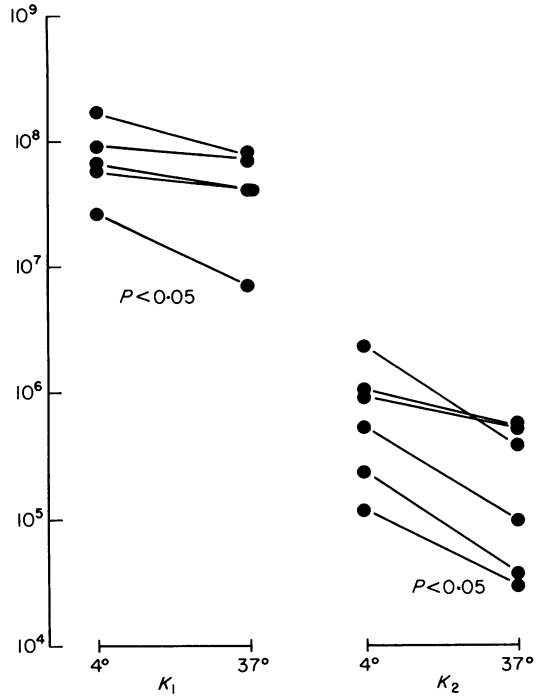

Figure 4. Equilibrium constants for complexes incubated at 4 or 37° after isolation. Complexes were harvested from ultracentrifuge gradient fractions 8–10. Incubations were for 24 h.

no difference was detected in K_1 for complexes formed in antigen or antibody excess ($P > 0.4$). Thus, antibody excess led to more stable complexes without affecting the stability of the binding of individual antibodies.

Effect of origin of complexes within the preparative gradient

Equilibrium constants K_1 and K_2 were compared for complexes isolated from fractions 8–10 of the preparative gradient and from fractions 11–13 of the same gradient (Ag_2 and Ag_1 complexes, respectively). Data are shown in Fig. 5. Larger complexes from fractions 3–5 were also analysed. Since the molecular formulae of these complexes could not be accurately determined, the data could only be expressed as percentage of antigen in the gradient appearing in the identical fractions from which it was pooled. Complexes were incubated after isolation at 4 or 37° for 24 or 72 h.

There was no difference for K_1 comparing Ag_2 and Ag_1 complexes ($P > 0.1$). K_2 was significantly greater for Ag_2 complexes than for Ag_1 complexes when all the data were pooled. There were insufficient data for separate analysis of 4 and 37° data. Selection of more stable complexes in the primary run appeared to apply also to larger complexes; from 21 to 33% of the total antigen applied was found in fractions 3–5, whereas negligible quantities of antigen were found in these fractions upon re-centifuging Ag_2 or Ag_1 complexes. Most of the complexes originally containing three to

Table 1. The effect of the initial antibody–antigen combining ratios upon equilibrium constants K_1 and K_2

	K_1		K_2	
	Antigen excess	Antibody excess	Antigen excess	Antibody excess
Combining ratio				
Mean	1.5×10^8	8.9×10^7	1.8×10^5	9.6×10^5
SEM	7.7×10^7	1.8×10^7	6.0×10^4	2.9×10^5
n	12		12	
	$P > 0.25$		$P < 0.01$	

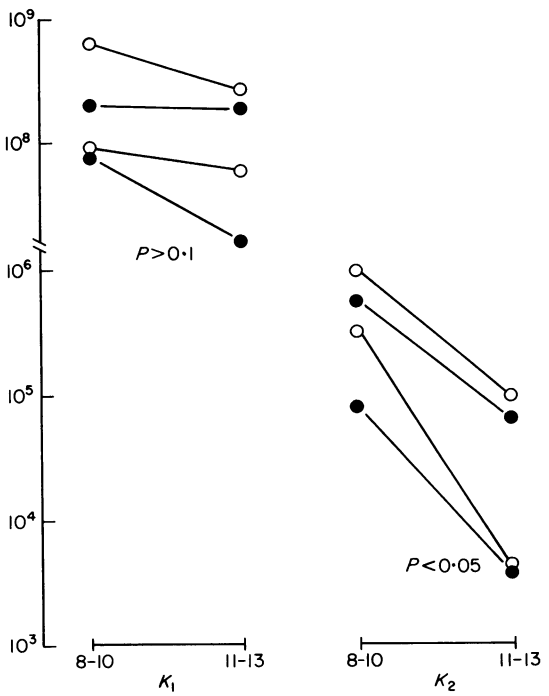

Figure 5. Equilibrium constants for complexes harvested from ultracentrifuge gradient fractions 8–10 and fractions 11–13. Isolated complexes were incubated at 4 (○) or 37° (●) for 24 h.

eight antigen molecules had, however, dissociated into Ag_2 and Ag_1 complexes. Thus, more stable complexes, but not higher affinity antibody, were selected by the primary ultracentrifuge run.

Effect of concentrations

The efect of concentration upon the stability of soluble immune complexes was assessed by comparing the equilibrium constants K_1 and K_2 for complexes isolated from concentrated preparations with those obtained from more dilute preparations but with the same antibody–antigen combining ratios (Fig. 6). Total protein in the 'concentrated' group ranged from 1·1 to 2·0 mg/ml and in the 'dilute' group from 0·1 to 0·6 mg/ml with a four- to ten-fold difference between concentration for each pair. The total protein applied to the secondary ultracentrifugation runs was also four- to ten-fold more for complexes isolated from 'concentrated' preparations compared with 'dilute'. Aliquots were incubated at 4 and 37° for 24 h. The data are shown in Fig. 6. K_1 was lower and K_2 higher for complexes formed and isolated at lower concentrations.

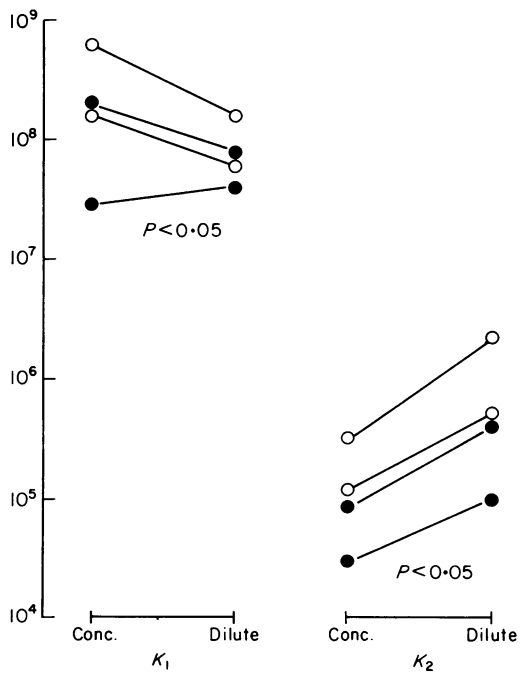

Figure 6. Equilibrium constants for complexes from fractions 8–10 formed and isolated in dilute and concentrated preparations. Isolated complexes were incubated at 4 (o) or 37° (•) for 24 h after isolation.

DISCUSSION

Studies concerning the mechanisms for the formation of various-size soluble immune complexes are often hampered by the fact that multiple association and dissociation reactions are occurring simultaneously, coupled with the inherent difficulty of measuring any one species in the presence of all the others. In this paper, we have characterized some of these reactions by studying the behaviour of simple, isolated complexes as analysed by ultracentrifugation. Complexes were formed between oligovalent fluorescein (F) conjugates of either rabbit serum albumin (3F-RSA) or thyroglobulin (3F-RTg) and rabbit anti-F antibodies. We observed that at the concentrations studied, 3F-RTg complexes originally containing two to eight antigen molecules when isolated tended to rearrange to form Ag_2 and Ag_1 complexes. Furthermore, isolated Ag_1 complexes tended to form some Ag_2 complexes and vice versa. Isolated complexes tended to generate only small quantities of unbound antibody. Most of our studies were therefore centered on the effect of various parameters on the interactions occurring among Ag_2 and Ag_1 complexes. Two equilibrium constants were derived from the Law of Mass Action for the special circumstances of these experiments. K_1 was used to measure the tendency for bound antibody to dissociate from complexes. K_2 measured the overall equilibrium reaction resulting in the relative proportions of Ag_1 and Ag_2 complexes. As envisioned by us, this second process occurred by the association and dissociation of subunits containing one antigen molecule and a mean number of antibodies determined by the original antibody–antigen combining ratio.

Values for K_1 ranged from 0·5 to 9 $\times$ 10^8 l/mol, and this generally agreed with the affinity constant for this pool of anti-F antibodies (2·1 $\times$ 10^8 l/mol) measured by the method of Stupp *et al.* (Stupp, Yoshida & Paul, 1969). K_1 exceeded K_2 in all experiments by approximately two orders of magnitude. The magnitude of this difference is quite striking when one considers that the forces cross-linking subunits of complexes are probably the same as those binding individual antibodies to antigen. In all likelihood, the distances between haptenic groups on the oligovalent and very large carrier thyroglobulin preclude the binding of more than an occasional antibody to two haptenic

sites on the same carrier. Thus, monogamous bivalency seems an unlikely explanation for the differences observed. More likely, the random distribution of haptens on the carrier produced some stress on the antibody–hapten bonds, especially when antigens were cross-linked at more than one site. Negative protein–protein interactions might also have contributed to the instability of complexes containing more than one antigen molecule. For K_1 to exceed K_2 does not appear to be specific for fluorescein since similar findings have been observed by us for isolated complexes prepared with dinitrophenol (DNP) conjugates and goat anti-DNP antibodies (Kijlstra, Knutson, van der Lelij & van Es, 1977).

The findings of higher equilibrium constants K_1 and K_2 at 4 than at 37° is not surprising; not all antigen–antibody reactions are thermolabile (Eisen, 1974). The current findings give quantification to the differences in thermolability for the reaction between antibody and its hapten and for the equilibrium reaction between Ag_2 and Ag_1 complexes. The inter-complex reactions were seen to be proportionately more thermolabile, which might be interpreted to mean that non-specific forces are relatively more important in the association and dissociation of Ag_1 and Ag_2 complexes. Such forces might include Fc interaction.

K_1 did not rise as a function of the original antibody–antigen combining ratio. One might have expected that in antibody excess there would have been competition for available hapten that would have favoured the selection of higher affinity antibodies. It is possible that the antibodies were relatively homogenous with regard to affinity. Alternatively, the ranges of antibody excess studied may simply have been insufficient to result in a detectable selection of higher affinity antibodies. In constrast, K_2 was higher for complexes formed in antibody excess than for complexes formed in antigen excess. Antibody excess complexes were relatively rich in antibodies as evidenced by their higher Ab/Ag ratios. This might provide greater opportunity for antigens to be cross-linked by more than one antibody molecule, thus forming a more stable ring structure, as prediced from thermodynamic considerations by Schumaker *et al.* (Schumaker, Green & Wilder, 1973), and demonstrated with electron microscopy (Valentine & Green, 1967; Hyslop, Dourmashkin, Green & Porter, 1970).

A similar explanation might account for the higher K_2 observed for complexes isolated as Ag_2 complexes compared with those isolated as Ag_1 complexes. In both cases equilibrium was established. Certain Ag_2 complexes might have been more stable because of fortuitous placement of haptenic groups or a selection of antigens containing more than the mean number of haptens since hapten conjugation to carrier probably has a Poisson distribution. Such antigens might allow cross-linking by multiple antibodies or some other more favourable steric configuration. Since K_1 did not differ for these same complexes, it seems unlikely that selection of complexes containing higher affinity antibodies contributed to the greater stability of Ag_2 complexes. It is not surprising that forming complexes in dilute solutions selects for more stable Ag_2 complexes; we have no explanation for the fact that K_1 was actually lower for dilute complexes.

Two experiments elucidated the kinetics of immune complex formation. In one experiment, K_1 and K_2 were compared for isolated complexes incubated 24 or 72 h. This showed that equilibrium had been achieved among isolated complexes. Association reactions would be expected to reach equilibrium within this time. Dissociation, however, might require longer time to reach equilibrium and so this experiment was necessary to validate the equilibrium constants K_1 and K_2. In the other kinetic experiment, the formation of complexes was shown to reach equilibrium within 1 h in that incubation of 24 h did not alter the ultracentifuge profiles of resultant complexes. More importantly, the stability of the resultant complexes was not increased by the longer incubation before isolation. Both observations support the conclusion that the most favoured configurations of soluble complexes, i.e. the lowest states of free energy, are reached within 1 h. It is not surprising that this would be true for antibody since available haptens are presumably similar in their binding properties so that the selective forces are small. It is somewhat surprising, however, that the more complicated interactions among complexes so quickly reach a steady state. Our explanation is that since K_2s are relatively low, the complexes dissociate rapidly enough for the selective forces to have exerted their influence by the time complexes are resolved from each other in the ultracentrifuge. Furthermore, antigens are oligovalent so the opportunities for more stable configurations are limited.

The experiments dealt mainly with equilibria established after isolation of soluble complexes. The results, however, may also be applicable to equilibria approached from the other side, i.e. the formation and growth of soluble complexes from antigen and antibody. The applicability is most clearly seen for complexes formed in relative hapten excess. Because the

association reaction for antigen and antibody is quite rapid and K_1 is relatively high, equilibrium is quickly reached with most antibodies being bound and the concentration of free antibodies sharply reduced within seconds. Assuming random distribution of hapten and random antibody binding to available hapten, most antibodies bind to form Ag_1 complexes. Complexes then form equilibria among themselves. In antibody excess states and at hapten–antibody equivalence, it is unlikely that appreciable amounts of free antigen are available. Therefore, complexes again probably grow by association of subunits. The ultimate size of complexes formed depends on many variables including non-specific protein–protein interactions (such as hydrophobic and ionic forces), steric factors, concentrations, and the Ab/Ag ratio of the subunit as limited by antigen valence and the antibody–antigen combining ratio. This last factor appears to play a determining role by affecting the ease with which subunits cross-link to form larger 'polymers'. It is possible that complexes also form and grow one molecule at a time as proposed in the reaction scheme of Steensgaard & Funding (1974). Both types of reactions undoubtedly occur (De Lisi, 1974). The K_2s which we measured were relatively low compared to K_1s. For this reason and for several other reasons given in van Es *et al.* (1979) it seems likely to us that complexes associate and dissociate mainly as smaller complexes rather than one molecule at a time.

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