and the number of times the α-particle strikes the barrier of the radioactive nuclides before it is expelled. The binding energy of about 1300 nuclei can be obtained by an empirical formula based on the above parameter. It is also possible to get the systematics of the resonance levels of light nuclei, as a process in which short-lived fundamental particles play the role of a virtual particle for the transfer of energy.

The relation between this method based on cardinality and the usual quantum mechanical approach has yet to be worked out. We, however, feel that there will not be any inconsistency as waves and particles are a special form of continuity and discreteness.

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Determination of kinetic parameters and thermodynamic constant of antigen–antibody reaction by solid phase binding method

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Study of the dissociation of [^{125}I]hCG from immobilized MAb–[^{125}I]hCG complex was carried out. The dissociation data fitted into a two-step reaction mechanism of the interaction. Kinetic parameters obtained were consistent, and were used to obtain the thermodynamic constants of the interaction. The first step (epitope recognition reaction) was shown to be a high-affinity intermolecular binding reaction involving epitope–paratope interaction, while the transformation reaction (second step) is an intramolecular rearrangement of the [^{125}I]hCG–MAb complex formed in the first step. The method described has the potential to be automated.

STUDY of real time kinetics of ligand–ligate interaction has greater information potential compared to equilibrium kinetics in understanding the chemistry of the interaction. However such kinetic study was rather difficult[1–4] until the advent of BIAcore or BIOS-1 (refs 5–7). Even though these methods are extensively used, they are expensive and suffer from uncertainties of interpretation[8,9]. We have described another method for these investigations using immobilized ligand and radioactive ligate[10,11]. This method though easy and cost effective, has the potential for further simplification. We present here an improved procedure, and have utilized these results to calculate the thermodynamic parameters of the MAb–[^{125}I]hCG interaction. The feasibility of automating the method is also discussed.

Materials and methods

Human chorionic gonadotropin (hCG) purified from early pregnancy urine[12] was used throughout the studies. Monoclonal antibody (MAb) VM-4 was raised against

hCG $\alpha\beta$ dimer and its characteristics have already been reported[10,13]. Microtiter wells and scintillation vials used were purchased from Plascis, Banglaore. All other reagents were of analytical grade.

Preparation of antimouse IgG-AH-sepharose

AH-Sepharose was obtained by treating epoxysepharose with diaminohexane[14]. AH-Sepharose (1 g in 5 ml) was treated with 10 µl of glutaraldehyde for 10 minutes, washed extensively with water and allowed to couple with IgG fraction of antimouse IgG at a protein concentration of 20 mg/ml (ref. 13). At the end of 20 h (at room temperature) more than 80% of the protein was coupled to the matrix. 50 µl of this matrix was found to have adequate capacity to adsorb the MAb from each aliquot.

Immunochemical bridge (ICB)

The method is the same as already reported[15,16], with slight modifications incorporated to improve the capacity for binding MAbs. The two types of ICBs investigated were the A bridge (Antimouse IgG adsorbed on plastic) and the AIA bridge (extended A bridge). Virgin plastic wells were coated first with antimouse IgG (15% sodium sulphate fraction) in water at 1/50 dilution (overnight at room temp.), washed and unbound sites blocked with bovine serum (2%). This represents the ICB-A for MAb adsorption. ICB-AIA was obtained by treating the ICB-A wells with mouse serum (1/100 dilution in RIA buffer for 6 h at room temp.) followed by antimouse IgG (1/100 dilution for 6 h at room temperature)[16]. The wells were drained, washed with RIA buffer and stored at 4°C until further use.

ICB in scintillation vial

Surface area to volume of a scintillation vial is too less and results in lower capacity to bind MAb. Hence surface area for adsorption of the antibody was increased by dropping a 1 cm length virgin plastic hollow cylinder (obtained by cutting virgin plastic tube of OD 0.9 cm and ID 0.6 cm). This effectively brings the surface area to volume close to that of the plastic well and results in binding of sufficient quantity of MAb. Scintillation vial with the hollow cylinder was coated with the ICB (using 2 ml of reagents at each step) and used for adsorption of the MAb throughout. The type of the ICB used was always the AIA type because of its greater capacity to bind MAb (see under results).

Antisera adsorption

Antisera at optimum concentration was added to scintillation vials (2 ml) or microtiter wells (250 µl) precoated with the ICB-AIA. Adsorption was allowed to occur at room temperature overnight and washed. These were stored at 4°C, and used within 15 days.

Binding of [^{125}I]hCG to immobilized antisera

Two ml of [^{125}I]hCG (50,000–100,000 cpm/100 µl) in RIA buffer (0.05 M phosphate buffer pH 7.4 containing 1% bovine serum) was added to scintillation vial precoated with the MAb. 250 µl of the radio label was used for binding to immobilized MAb in the microtiter well. Binding was allowed to occur overnight, washed, counted and dissociation started immediately.

Dissociation of [^{125}I]hCG from the MAb–[^{125}I]hCG complex

2.5 ml of RIA buffer containing 2 µg/ml unlabelled hCG was added to preformed [^{125}I]hCG–MAb complex in the scintillation vial. At intervals of time periods (ranging from 5 minutes to 60 minutes), 300 µl of the dissociating solution was transferred to a tube and the radioactivity measured in a LKB multigamma counter. At each point of time the background counts of the tubes was subtracted from the counts of the dissociation solutions to quantify the release of radioactivity accurately. The solution was then transferred *in toto* to the scintillation vial. The loss of volume of the solution from the start of the dissociation to the end of the dissociation was less than 5%. For studies with extended times the dissociation solution in the vial was kept in a closed water bath at the required temperature to eliminate losses due to evaporation.

Theoretical fit of the data

The dissociation has been shown to be a two-step reaction[10]

$$\text{MAb}+[^{125}\text{I}]\text{hCG} \leftrightarrow \underset{k_{-1}}{\text{MAb}-[^{125}\text{I}]\text{hCG}} \leftrightarrow \underset{k_{-2}}{\underset{(S)}{\text{MAb}-[^{125}\text{I}]\text{hCG}^*}},\ \underset{(VS)}{}$$

where MAb–[^{125}I]hCG represents the antigen–antibody (Ag–Ab) complex. The slow dissociating complex is identified as (S) and the very slow dissociating complex as (VS) for subsequent discussions. k_{-1} and k_{-2} correspond to the dissociation rate constants of the S → [^{125}I]hCG and the transformation of (VS) → (S) respectively.

The dissociation pattern was treated as a two-step consecutive reaction, the transformation of VS to S and dissociation of S to unbound (free) [^{125}I]hCG. The radioactivity measured in dissociation is the unbound [^{125}I]hCG. Those associated with S and VS are on the solid phase and are not released into the medium during

dissociation. Before the start of the dissociation, the solid phase complex consists of varying proportion of S and VS and had little unbound [^{125}I]hCG (0 time radioactivity 300 cpm). Dissociation of S as well as transformation of VS to S with its subsequent dissociation to free [^{125}I]hCG occurs during dissociation experiment. The reverse reactions of unbound [^{125}I]hCG rebinding to the immobilized MAb do not occur as dissociation was carried out in the presence of 1000-fold excess of unlabelled hCG. Thus the sum of radioactivity released (x) from S and VS at any point of time is

$$x = a_1[1-\exp^{(-k_{-1}t)}] + a_2[1-\exp^{(-k_{-1}t)}]*[1-\exp^{(-k_{-2}t)}],$$

where a_1 and a_2 are the radioactivity associated with S and VS at the start of the dissociation and t is time. Approximate value of the a_1, a_2, k_{-1} and k_{-2} are obtained from the extension of the linear part of the graph as described[10]. The total radioactivity dissociated ($a_1 + a_2$) is an experimentally-determinable quantity and becomes a precondition for changing other variables. Using these values, theoretical plot of dissociation data were obtained. The theoretical dissociation curve will fit into the experimental data with only one set of values of a_1, a_2, k_{-1} and k_{-2} which can be arrived at by iteration procedures, for an experimentally determined value of ($a_1 + a_2$). A fit of the experimental dissociation data and its convergence validates the two-step mechanism proposed and allows accurate quantification of rate constants k_{-1}, k_{-2}, and concentrations of S (a_1) and VS (a_2) forms of the complex at equilibrium.

Determination of affinity constants from dissociation data

Affinity constants in the two steps of the reaction were calculated using the equilibrium values of [S] (concentration of the S form of the complex), [VS] (concentration of the VS form of the complex), by the following formulae

$$K_{a1} = [S]/\{[C_{MAb}]-[S]-[VS]\}*\{C_{hCG}]-[S]-[VS]\} \text{ and}$$

$$K_{a2} = [VS]/[S]$$

with C_{hCG}, and C_{MAb} being the initial concentrations of the [^{125}I]hCG and MAb.

Determination of thermodynamic constants

Binding as well as dissociation experiments were carried out at different temperatures and the data were utilized to obtain accurate values of rate constants and equilibrium ratios. The thermodynamic constants were calculated using the following formula:

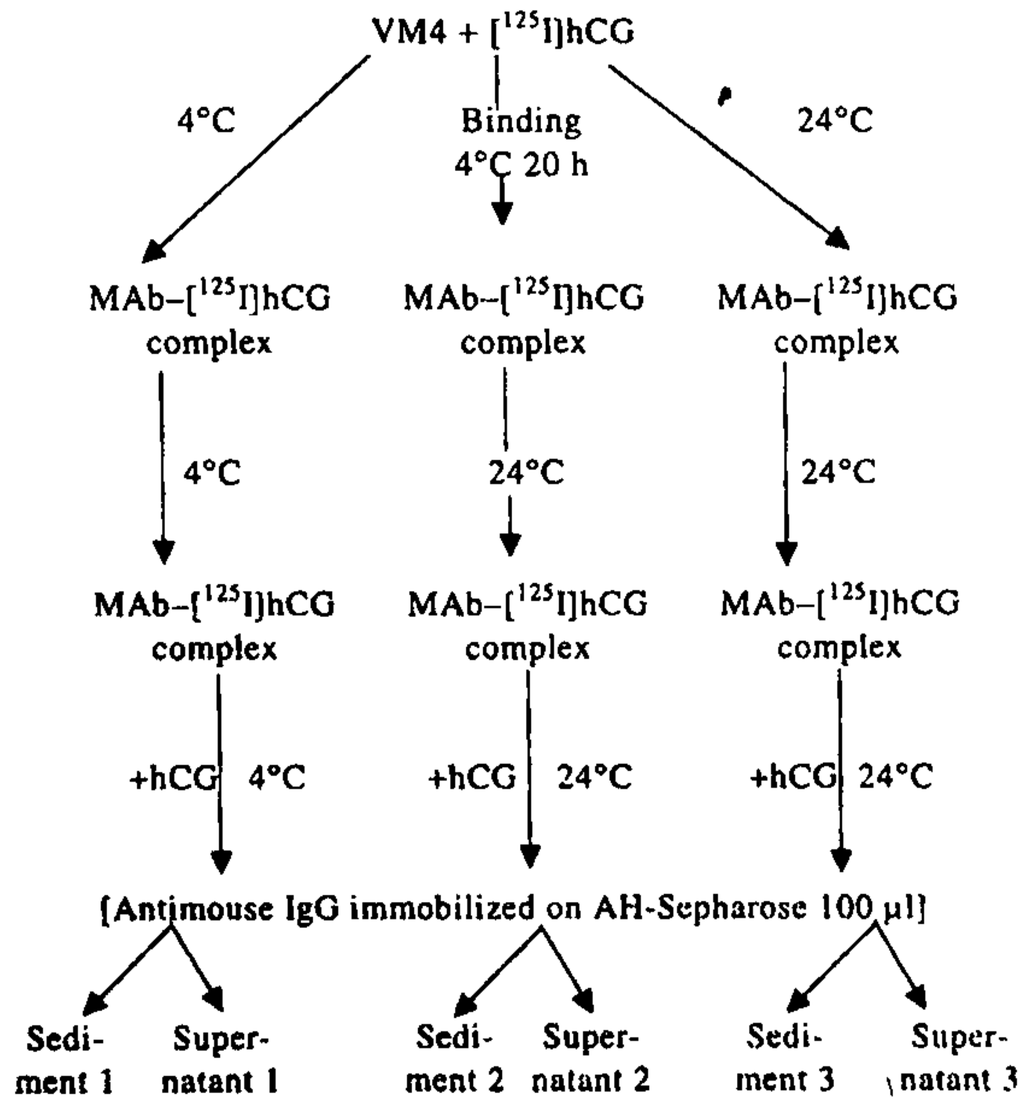
$$\Delta G = -RT \ln K_a.$$

ΔH was calculated by the equation

$$\Delta H = T_1T_2\,[R \ln K_{t1} - R \ln K_{t2}]/[T_2 - T_1],$$

where T_1, T_2 correspond to two temperatures, K_{t1} and K_{t2} are equilibrium constants between VS and S at these two temperatures. [(VS)/(S)].

Table 1. Schematic diagram for quantifying the reversibility of the transformation



Measurement of nonreversibility of MAb–hCG reaction in the liquid phase at low temperature

The rationale of the approach is shown in Table 1. MAb and [^{125}I]hCG were incubated in liquid phase in three parts (step 1). The first two parts were incubated at 4°C while the third one was incubated at room temperature. After overnight reaction, the first aliquot was maintained at 4°C while the 2nd was warmed to room temperature for dissociation to occur. The third aliquot remained at room temperature. After overnight incubation, unlabelled hCG in sufficient excess (20 µg/aliquot) was added to all the aliquots, and dissociation allowed to occur at the same temperature (1st aliquot 4°C: 2nd and 3rd aliquot room temperature). After further 20 h reequilibrium [^{125}I]hCG–MAb complex was removed from the reaction mixture of [^{125}I]hCG and MAb using immobilized antimouse IgG on AH-sepharose. For this purpose, sufficient excess of antimouse IgG-AHS matrix was added to each aliquot, vortexed for 10 minutes and unadsorbed radioactivity was measured in a multigamma counter (LKB). The extent of transformation of the nonreversible complex on warming to room temperature was quantified by the difference in the radioactivity between the supernatants of 1st and 2nd aliquots.

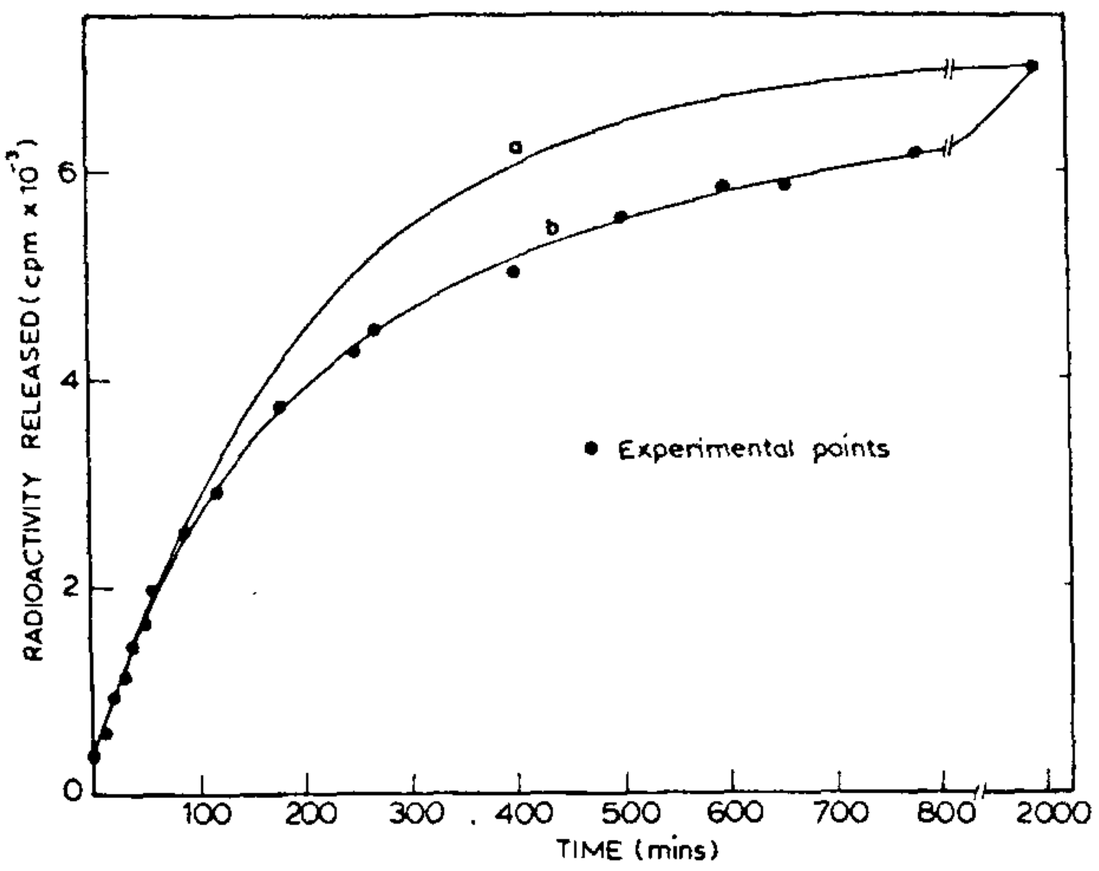


Figure 1. Dissociation profile of immobilized [^{125}I]hCG–VM4 complex in the presence of 2 µg/ml unlabelled hCG. Curves a and b present the theoretical graph for dissociation for single step (a) and two step (b) dissociation mechanism. Points represent the experimental data. In three separate experiments carried out at room temperature, the ratio of VS/S was 1.03 ± 0.15, k_{-1} and k_{-2} were 0.008 ± 0.0008/min and 0.001875 ± 0.00076/min respectively.

Results

The binding of [^{125}I]hCG was carried to immobilized MAb in microtiter wells as described under methods. Binding of [^{125}I]hCG to immobilized MAbs through the two types of ICBs showed better binding in the AIA type of immobilization (Table 2). This data clearly indicated that the capacity of the AIA type of ICB is much higher. In fact the extent of increase in the capacity was of the order of 5–10 fold determined by the Scatchard plot analysis of the SS-SPRIA done using these immobilized MAbs (data not shown).

The dissociation profile of [^{125}I]hCG-VM4 (Figure 1) showed that at start of dissociation the unbound [^{125}I]hCG was insignificant (<300 cpm), and with time it increased and became significant within 10 minutes (>200% of nonspecific value). Hence analysis of the data at early dissociation periods could be carried out with greater accuracy than by the one reported earlier[10]; the latter method based on the measurement of the retention of the radioactivity on the microtiter wells. Dissociated radioactivity at all points of time was found to be adsorbed completely on to immobilized VM4 to >95% and hence ruled out the dissociation to be the result of the damage of the [^{125}I]hCG in the complex due to radioactive decay.

The fit of the experimental data (closed points) to theoretical curve (Figure 1, curve b) of the dissociation

Table 2. Binding of [^{125}I]hCG to immobilized MAbs through different immunochemical bridges

MAb immobilized*	ICB-A	ICB-AIA
VM14	9500	15100
51-16	7800	14000
51-21	2000	7000
52-28	8500	33000

*All MAbs were immobilized at saturating concentrations. Microtiter wells were incubated with 100,000 cpm of the [^{125}I]hCG in 250 µl for overnight.

substantiates the two-step mechanism proposed for the antigen–antibody interaction. The values obtained for k_{-1}, k_{-2}, a_1, a_2 were consistent over several determinations with an error of not more than ± 15% (Table 3). The values obtained were also independent of the starting concentration of the complex. This consistency of the proportions of the (S) and (VS) forms has made it possible for us to use these values for the determination of the thermodynamic constants of the reactions at the two stages.

Best fit values of dissociation data obtained at three temperatures (Table 4) showed that the proportion of S/VS increased with temperature. The extent of radioactivity which remained bound was high (85%) at 4°C, and was 18% and 12% at 22°C and 34°C respectively. The thermodynamic constants calculated (Table 5) indicated that the primary reaction was associated with large free energy change (ΔG = 12 kcal),

Table 3. Determination of kinetic parameters from dissociation data

Expt no.	[^{125}I]hCG added (cpm $\times 10^{-5}$)	[^{125}I]hCG bound (cpm $\times 10^{-5}$)	a_1 (cpm $\times 10^{-3}$)	a_2 (cpm $\times 10^{-3}$)	a_1/a_2	k_{-1} per minute	k_{-2} per minute
1	21.0	3.20	23.0	18.0	0.80	0.0075	0.00030
2	22.0	1.57	10.0	11.0	1.10	0.0070	0.00020
3	15.0	0.55	3.3	3.5	1.05	0.0090	0.00016

The coating conc. of the MAb was 1/1 k, 1/5 k and 1/10 k respectively.

Table 4. Kinetic constants of VM4-[^{125}I]hCG dissociation obtained by theoretical fit of the experimental data

Temperature (°C)	4	22	34
Bound a_1 (cpm)	–	2100	3300
k_{-1} (units/min)	–	0.0085	0.0090
a_2 (cpm)	–	8100	3500
k_2 (units/min)	–	0.0009	0.0016
% undissociated	85	18	12

Input of radioactivity for binding was 14,00,000 cpm in 2.5 ml at respective temperatures.
%Undissociated is the % radioactivity still bound after 24 h incubation with unlabelled hCG.

Table 5. Thermodynamic parameters of VM4–hCG interaction

	Temperature (°C)	K_a(lM^{-1})*	ΔG (cal/mole)	ΔH (cal/mole)	ΔS (cal/deg.mole)
Step 1	22	4.37×10^8	11664	–	+23.5
(Binding)	34	7.42×10^8	12461	4736	+25.1
Step 2	22	3.81	–780	–	–62.4
(Transformation)	34	1.06	–35	19178	–62.3

*The affinity constants calculated as detailed under methods.
$C_{MAb} = 0.3445 \times 10^{-10}$ M/l.

Table 6. Reversibility of transformation in liquid phase

	Radioactivity (cpm) 4°C	4°/24°C	24°C
Sediment	46000	2000	3000
Supernatant	4000	50900	41500
% dissociated in liquid phase	8	96	93
% dissociated in solid phase	15	–	88

The total binding was 90% before dissociation was started. All values are normalized to 100% bound by deleting the contribution of the 10% unbound [^{125}I]hCG. % dissociation in the solid phase is obtained from the dissociation data (Figure 1).

while the transformation reaction had a large change in enthalpy ($\Delta H = 19$ kcal). The entropy changes calculated showed a change of +25 cals/degree.mole during the (S) complex formation and –62 cals/degree.mole in the transformation (S → VS). Equilibrium values calculated for the first step of the interaction (Ag + MAb → S) showed the affinity constant for this step to be lower by 80% than the average affinity constant measured by Scatchard plot analysis using the SS-SPRIA [3×10^9 (ref. 10)], and was also less dependent on temperature than the S → VS transformation.

Both kinetic and thermodynamic data demonstrated structural changes of the immobilized [^{125}I]hCG–MAb complex with time. It also indicated an increase in the proportion of the VS/S at low temperature (Table 4), with < 10% dissociation occurring at 4°C of the immobilized complex in microtiter wells (data not shown). This transformation inferred is not an artifact of the solid phase was proved by the demonstration of the apparent nonreversibility of the complex at 4°C in the liquid phase (Table 6). Preferred complex at 4°C failed to dissociate (step 1), while the same complex dissociated to 96% after warming to room temperature (step 2) comparable to that of the complex formed at room temperature (step 3). This also indicated that the transformation is indeed reversible as observed in the solid phase[10]. The extent of dissociation observed from the immobilized VM4–[^{125}I]hCG complex is comparable in the solid and liquid phase dissociations (Table 4 and Table 6).

Discussion

Extension of the ICB resulted in increased capacity to bind MAbs. In fact, capacity determined by Scatchard analysis of SS-SPRIA of such microtiter wells indicated an increase in the capacity of 5–10 fold both in MAbs as well as in polyclonal antibodies (data not shown). Thus extending the ICB allows efficient utilization of MAbs, especially low-affinity antibodies in investigations which use solid phase binding approaches.

Mechanism of the Ag–MAb reaction is a multistep process involving transformation and indicates that other macromolecular interactions could be complex. The first step of the interaction is tentatively attributed to epitope–paratope interaction (recognition), while the second step is an intramolecular arrangement of the complex for greater thermodynamic stabilization. Several MAb–[^{125}I]hCG pairs indeed showed this pattern and hence indicate the possibility of multiple-step mechanism in other macromolecular reactions like those of hormone–receptor, lectin–carbohydrate, etc. Its relevance in the biological processes needs to be

investigated to quantify the importance of the second slow transformation.

The dissociation profile can fit possibly to two-step independent dissociations. This possibility is unlikely on the grounds that this molecular interactions is extremely specific, and the study of the MAb with antigens is always considered homogeneous. In addition, such a possibility appears unlikely for the following reasons. The MAb antigen reaction is extremely specific ligand–ligate system and hence the first reaction is the formation of the Ag–MAb complex, which is identical no matter which of the paratope of the MAb binds to the antigen. A second complex can be obtained when the Ag–MAb complex binds further at the second paratope of the MAb to form a complex of the type (Ag–MAb–Ag). The formation of such a complex is unlikely under the experimental conditions since the ratio of the Ag bound is <10% of the antibody immobilized. In addition, the [^{125}I]hCG–MAb complex gets transformed in the absence of unbound [^{125}I]hCG (ref. 10). Rate constants and the ratios of S/VS are not influenced by the specific binding and hence do not favour the formation of (Ag–MAb–Ag) type of complex. Should such a complex be formed, its dissociation pattern would not be sum of two independent dissociations, and would follow a far more complex kinetics. Furthermore, binding of Ag at one paratope does not appear to affect the binding at the other paratope as Scatchard plot analysis failed to show a second binding constant in several MAb SS.SRIAs (unpublished observation). Thus the possibility of two independent dissociations appears unlikely.

Since enthalpy changes in protein folding/unfolding have been attributed to changes in the accessible polar and apolar .surface area associated with the folding process[17–22], a large change in ΔH seen in the S → VS transformation indicates marked difference in the accessible polar surface area during the transition. Thus hydrophobic bonding forms major force in the transformation process in this system. The change in entropy is –62 cals/degree during the S → VS transformation, almost completely contributed by the enthalpy (Table 5). This, based on calculations of the thermodynamics of denaturation of proteins indicates reordering of a minimum of 15 amino acid residues between the MAb and hCG during the S → VS transformation (4 cals/residue/K)[17]. Thus it is probable that at least 15 amino acid residues (consisting those of hCG and the antibody) are involved during the transformation process. This appears to match well with the magnitude of the epitope as deciphered by epitope analysis[12,23–25]. However, the entropy change in the first step of the reaction is not exclusively due to the enthalpy change (ΔF = 12 kcal/mole), and hence does not allow any conclusion to be drawn about the nature of this interaction.

The equilibrium being affected by the presence of the cold hCG is also unlikely. Proportion of dissociation is independent of the absolute quantity of the complex, and hence formation of the hCG–MAb complex does not change the % dissociation. Thus the radioactivity released is independent of the formation of large quantity of hCG–MAb complex during the dissociation of [^{125}I]hCG–VM4 complex.

The data also shows the importance of the carrier used for coupling of peptides for immunization. It is clear from these studies that for the epitopic region of VM4, a carrier like hCG molecule itself provides an increased binding activity due to secondary reaction with the MAb. It was also seen that in several MAbs presence of the C-terminal region obstructs the stabilization of the complex (unpublished observations). This indicates a major role for the carrier in eliciting right antibody response, and the method described here can be utilized to identify appropriate macromolecule for conjugation. In expression of bioactivity slow transformation is indeed important as the VS type may form significant part of the Ag–Ab complex in circulation and may determine the efficacy of the antisera. Identification of the transformation reaction also indicates the possibility that a small molecular weight drug may exhibit enhanced activity on coupling to an appropriate nonspecific protein because of the stabilization of the [protein–drug]–receptor complex following recognition of the drug. Thus measurement of these thermodynamic constants can provide useful data in the design of synthetic drugs.

The thermodynamic constants for most of the antigen–antibody reactions have so far been determined by microcalorimeter. Since macromolecular interactions appear to involve subsequent transformations, it would be interesting to see how the results obtained by microcalorimetry compare with those obtained by the realtime kinetic approach as described above.

The method described in this paper has several advantages over the earlier method[10]. In this method the radioactivity measured at zero time is very small and the increase in the radioactivity in a 10-min period is several-fold more. This allows accurate analysis of the initial part of the dissociation. Operationally, this method is far simpler than the other method, requiring a single scintillation vial for binding and dissociation. Because of the consistency observed in the kinetic parameters, they can be used for calculating thermodynamic constants which are very crucial for understanding the structural aspects of macromolecular interactions. This method perhaps will be the least expensive method for determination of thermodynamic constants in protein–protein interaction. The method is also amenable for automation. It is possible to envisage the complete automation of the kinetic investigations, providing a viable inexpensive alternative for the

BIAcore. Further work is in progress towards this end. The woes of radioactive disposal do not arise since the total disposal of radioactivity is restricted to a single scintillation vial in each experiment.

Kinetic parameters and thermodynamic data obtainable from the above approach are based on solid phase dissociation. How far these figures match with values that can be obtained in the liquid phase reaction is unknown. The values may not be very different is indicated by the observation that the extent of dissociation of the MAb–[^{125}I]hCG complex in solid phase and liquid phase were comparable at low and high temperatures (Table 6, Table 4). Despite these limitations and in absence of a convenient method to determine these parameters in liquid phase, the above approach provides an easy method for obtaining the thermodynamic data from the kinetic parameters of the dissociation reaction. It may also be indicated here that thermodynamic constants have indeed been reported extensively using BIAcore system which is also a solid phase approach[26,27]. The objection that radiolabelled hCG differs from unlabelled hCG is unlikely in view of the observations that radiolabelled and unlabelled proteins behave similarly in a BIAcore dissociation. Similarly extent of loss of epitopic activity of hFSH to modifications is same whether the modification is done using radiolabelled or unlabelled FSH (ref. 25). Extensive chemical modification of hCG has failed to show significant structural change at majority of the epitopic regions[13]. These data suggest strongly that iodination of hCG does not cause structural changes at the epitopic regions, and hence results obtained are not artifacts of iodination of the ligand.

In summary, a method was developed to determine the real-time dissociation constants of [^{125}I]hCG–VM4 complex. Based on these data the antigen–antibody reaction was deduced to consist of two steps, for which thermodynamic parameters were also obtained. This method promises to be an inexpensive alternative for determining the real-time kinetic parameters of ligand–ligate interaction.

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