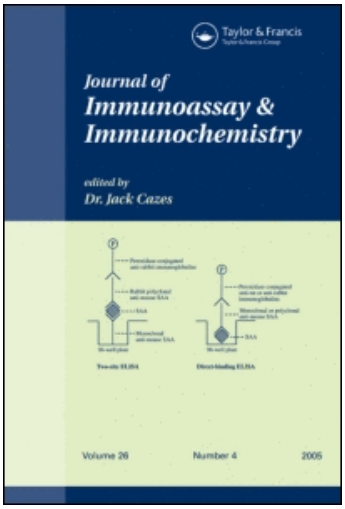

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THE EFFECTS OF PEG ON SECOND ANTIBODY IMMUNOPRECIPITATION AND ITS USE IN IMMUNOASSAY

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ABSTRACT

The effects of PEG on the second antibody immunoprecipitation have been studied in several radioimmunological systems. The following parameters have been studied in the assays: the average molecular weight and concentration of PEG, the species of the animal producing the first and second antibody, the nature of the antigen, the concentration of carrier immunoglobulins, and the rate of the immunoprecipitation. Thus, optimal conditions for the use of PEG with a second antibody have been defined in order to be used in any liquid phase immunoassay.
(**KEY WORDS:** double antibody, polyethylene-glycol, immunoprecipitation, immunoassay)

INTRODUCTION

We present here a study of the effects of PEG on second antibody separation using several radioimmunological systems including haptens or high molecular weight antigens. The effects of the following parameters on the separation process have been studied:

- the nature and concentration of PEG,

- the animal species in which the first and the second antibodies are raised,

- the nature of the antigen and the hapten,

- the concentration of carrier immunoglobulins.

This study has also been undertaken in order to define optimal conditions under which PEG can be used in conjunction with a second antibody to improve the separation process in heterogeneous competitive immunoassays.

MATERIALS AND METHODS

Reagents

Chemicals were purchased from Merck, Darmstadt, Germany, in particular PEG 200 (mean Mr: 190-210), 600 (570-630), 1500 (1400-1600), 6000 (5000-7000) and 10000 (8500-11500).

Rabbit antisera against human growth hormone (hGH), cyclic AMP (cAMP) and guinea-pig antiserum against insulin were purchased from ERIA-Diagnostics Pasteur, Marnes, France. Rabbit antiserum against human prolactin (PRL) was kindly supplied by Dr Dray, Paris. Antisera against type I and IV collagens (1,2), acetylcholinesterase (AChE) (from electric organs of Electrophorus Electricus) (3), substance P (4) and dihydroergocristine (DHECT) (5) were raised in rabbits or guinea-pigs as previously described. Second antibodies against human, rabbit, guinea-pig, mouse and goat immunoglobulins G were obtained by immunization of swine, goat, horse and

rabbit with IgG purified by DEAE chromatography, using standard immunization procedures (6).

Tyrosine methylester-monosuccinyl-cAMP (Sigma, St Louis, USA), substance P (kindly supplied by Dr Regoli, Sherbrook, Canada), porcine insulin (Novo, Copenhagen, Denmark), hGH and PRL (prepared by Dr Dray), AChE, human type I and IV collagens (supplied by Dr Herbage, Lyon), DHECT (Rhône-Poulenc, Vitry, France), honey-bee venom (Pharmacia, Uppsala, Sweden) and a monoclonal mouse IgG (IgG1, K) were labelled with iodine 125 and purified using established procedures (7). The characteristics of the different tracers used in this study (molecular weight, specific radioactivity) are given in Table 1.

Radioimmunoassay and Second Antibody Immunoprecipitation

All experiments were performed in 2 steps, in (5 x 1 cm) polystyrene tubes using the following buffer: 0.05 mol/L sodium phosphate, 0.15 mol/L NaCl, 0.02 % Tween 20®, 0.01 % sodium azide, pH 7.4. In the first step, a predetermined dilution of the first antibody was allowed to react for 24 h with the labelled antigen or hapten (5000 to 20000 dpm). The first antibody dilution was calculated in order to precipitate, under optimal conditions, 30 to 60 % of the total radioactivity introduced in the assay (Table 1). In the second step, the second antibody and PEG (or buffer) were added to precipitate the antigen-first antibody complexes. In most of the experiments, non-immune serum from the species in which the first antibody was raised was

TABLE 1

Characteristics of the Different Assays

Assays	Mr (Da)	Tracer specific radioactivity	First antibody		References
			Species	Dilution	
Human growth hormone	22000	150 Ci/g	rabbit	1:60000	*
Human prolactin	24000	100Ci/g	rabbit	1:45000	*
cAMP	329	1500 Ci/mmol	rabbit	1:20000	*
Substance P	1350	1500 Ci/mmol	rabbit	1:40000	(4)
Dihydroergocristine (DHECT)	700	1500 Ci/mmol	rabbit	1:40000	(5)
Acetylcholinesterase (AChE) G4 A8 A12	320000 796000 1150000	6 Ci/g	rabbit	1:600000	(3)
Mouse laminin	850000	8 Ci/g	rabbit	1:50000	(11)
Type I collagen	330000	10 Ci/g	goat	1:100	(1)
Type IV collagen	540000	10 Ci/g	guinea pig	1:500	*
Type IV collagen	540000	10 Ci/g	rabbit	1:20000	(2)
Type IV collagen	540000	10 Ci/g	mouse	1:1000	*
Anti-insulin IgG	154000	200 Ci/g	human	1:3	(9)
Anti-honey bee venom IgG	154000	2 Ci/g	human	1:600	(10)

* unpublished experiments

added as an immunoprecipitation carrier in order to obtain a macroscopic precipitate. Depending on the experimental procedure (see below), these non-specific immunoglobulins were added during the first or second incubation period. The first step was achieved in two ways:

- The first antibody and the tracer were mixed in a total volume of 0.3 mL containing 0.1 mL of diluted antiserum, 0.1 mL of tracer and 0.1 mL of either diluted non immune serum or buffer. Non specific binding was measured by replacement of the first antibody by buffer. Tubes were incubated at 4°C or room temperature for 24 h.

- In order to avoid loss of time due to the first incubation, we used an equivalent bulk procedure. Large volumes (50 to 200 mL) of the first antibody and the tracer were incubated together for at least 48 h at either 4°C or room temperature. At the end of this first incubation period, 0.2 mL of the mixture was added to 0.1 mL of non-immune serum or buffer and the immunoprecipitation was continued as described below. Antiserum and tracer dilutions were calculated to give concentrations (in 0.2 mL) equivalent to those used with the first procedure. Depending on the radioimmunological system tested, these large volumes of prereacted reagents could be used for 2 to 6 weeks without significative changes in the final result. Theoretically, these two procedures differ in the duration of the first incubation step and in the presence or absence of non-immune serum during incubation. In practice, they gave identical results. The only difference was noted in acetylcholinesterase assay for which higher non-specific binding was observed

when the first procedure was used. This behaviour was probably due to non-specific interaction between asymmetric forms of the enzyme and uncharacterized components of non-immune serum, as it has been previously suggested (8).

During the second step, at the end of the first incubation, the complexes were precipitated by adding either 0.1 mL of PEG at different concentrations or buffer, followed by 0.1 mL of a predetermined dilution of the second antibody, so that the final immunoprecipitation occurred in a total volume of 0.5 mL in both procedures. The final mixture with PEG was further incubated for 2 h at room temperature. In the absence of PEG, incubation of 6 h was used, since preliminary experiments had shown that these incubation times resulted in virtually complete immunoprecipitation whatever were the experimental conditions (temperature, concentration of PEG, ...) with the selected batches of second antibodies.

In some experiments (see Fig. 4), immunoprecipitation of the first antibody was directly monitored by using ^{125}I-labelled immunoglobulins (mouse monoclonal antibody γ1, K) (as antigen) and rabbit anti-mouse IgG antibody. In this case, the first step was eliminated and the second step was performed as already described (total volume 0.5 mL). The following reagents were mixed: 0.1 mL labelled IgG, 0.1 mL buffer, 0.1 mL non-immune serum (or buffer), 0.1 mL 10 % PEG (or buffer) and 0.1 mL second antibody. At the end of the second incubation period, 2 mL of buffer were added to each tube and the tubes were then centrifuged at 2500 x *g* for 30 min. Supernatants were

discarded and the radioactivity of the pellets was measured in a solid scintillation counter (LKB Multigamma, 78 % counting efficiency). All measurements for both specific and non-specific binding were performed in duplicate. Non-specific binding was substracted from all experimental values and the results were expressed as a percentage of the maximum precipitation observed. The concentrations and dilutions shown in all figures refer to the initial 0.1 mL volume before addition to the other reagents.

Kinetic studies

We used the following procedure to study the time course of immunoprecipitation: all first-step reagents were reacted and then the second antibody and PEG (or buffer) were added at different time intervals; 2 mL of buffer were then added to all tubes and the reaction was allowed to continue as described above. The times indicated on the curves refer to the periods between addition of the second antibody and the beginning of centrifugation. Since the kinetics of the reactions with second antibodies are in general rapid even in absence of PEG (6 h or less), we chose to lower them by performing the incubations with a less powerful second antibody (batch of goat anti-rabbit IgG antibody which could be diluted only twice).

RESULTS

Shape of the Precipitation Curves in the Presence or Absence of PEG

We first studied the influence of PEG 6000 on the shape of the

precipitation curves. In these experiments, the concentration of second antibody was constant and the amount of the first antibody (and thus of immunoglobulins G) precipitated was indirectly determined by measuring the quantity of radioactive antigen (or hapten) present in the immunoprecipitate (see Methods). We used the classical representation for antigen-antibody precipitation curves by plotting the precipitated radioactivity against the concentration of carrier immunoglobulins introduced in the assay, which are considered to be the antigen for the (second) antibody.

In the absence of PEG (Fig. 1), the curves exhibit a characteristic form with three distinct zones corresponding to an excess of antigen, an equivalence between antigen and antibody, and an excess of antibody. The presence of increasing concentrations of PEG 6000 resulted in a broadening of these curves which was more pronounced in the excess antibody zone. As a consequence, the zone for which maximum precipitation occurred was greatly extended. In addition, in the substance P assay (Fig. 1) we observed with PEG some increase in the radioactivity precipitated at this maximum value.

Whatever the nature of the antigen of PEG, and of the first or second antibody, the whole curves were qualitatively the same, but quantitative differences were noted. We therefore undertook a more detailed study of the different parameters affecting second antibody immunoprecipitation.

Influence of the Species producing the Second Antibody

We compared the properties of various second antibodies directed against rabbit, guinea-pig, goat, mouse, and human IgG raised in different species:

rabbit, swine, goat, and horse. They all provided nearly identical precipitation curves, except for horse second antibody. This is illustrated in Figures 1A and 1B, which show the curves obtained with labelled substance P, rabbit anti-substance P, and either swine (Fig. 1A) or horse (Fig. 1B) second antibody. In the absence of PEG, the horse second antibody gave a sharper curve with a narrower equivalence zone when compared with all other second antibodies tested. In the presence of PEG, there was a broadening of the precipitation curves so that all second antibodies produced by the different species, including the horse second antibody, appeared to work in a similar manner.

Influence of the Species producing the First Antibody

In this study we used 13 different first antibodies raised in five different species: rabbit, guinea-pig, goat, mouse, and man (Table 1).

We never observed significant differences in the precipitation curves obtained when first antibodies from different species were used with a second antibody raised in a single species (not shown).

Influence of the Nature of the Antigen

We have tested eleven different haptens or antigens including low molecular weight haptens (cAMP, DHECT, venom components), peptides (substance P), hormones (insulin, growth hormone, prolactin), and high molecular weight proteins (collagens, AChE, laminin) (see Table 1). Without PEG, we obtained similar precipitation curves, whatever the antigen. In the

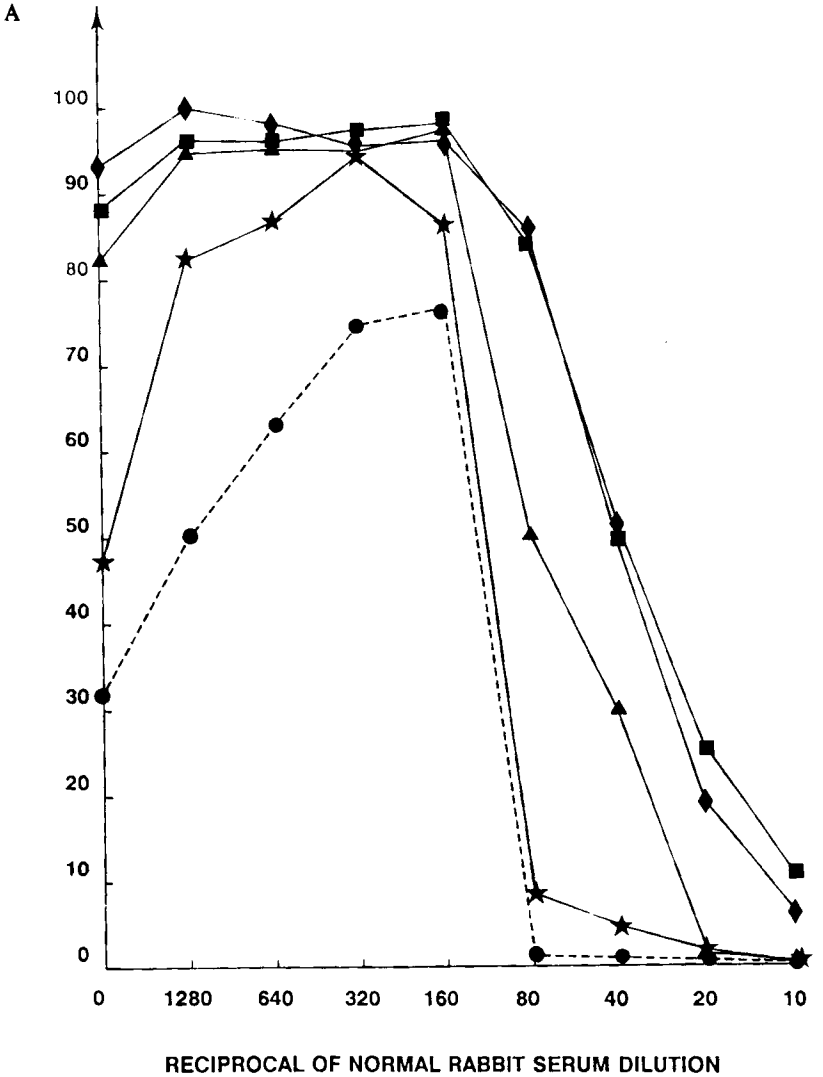

FIGURE 1: Influence of PEG 6000 on the shape of precipitation curves. The percentage of maximum precipitation of substance P bound to first antibody is plotted against the reciprocal of the normal rabbit serum dilution for different PEG concentrations: 0 % (●), 1 % (★), 2.5 % (▲), 5 % (◆) and 10 % (■). Second antibody was swine (1A) or horse (1B) antibody diluted 4-fold. 100 % precipitation corresponds to B/T = 49 % (non specific binding < 5 %).

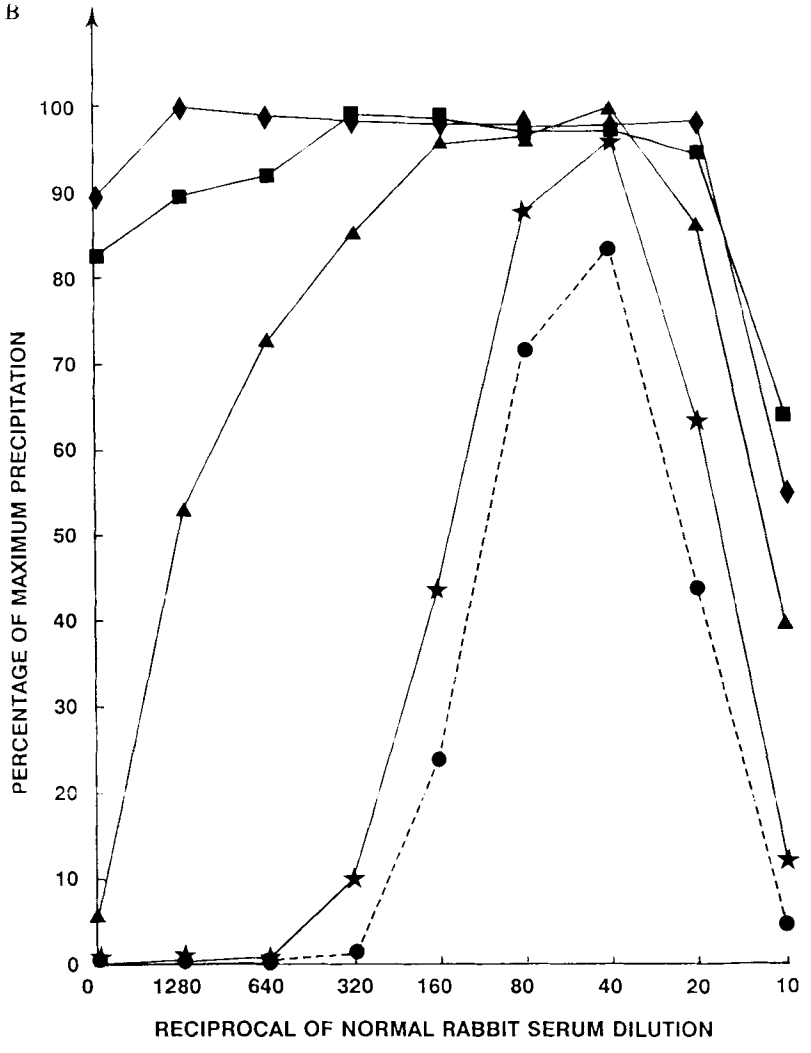

FIGURE 1 Continued

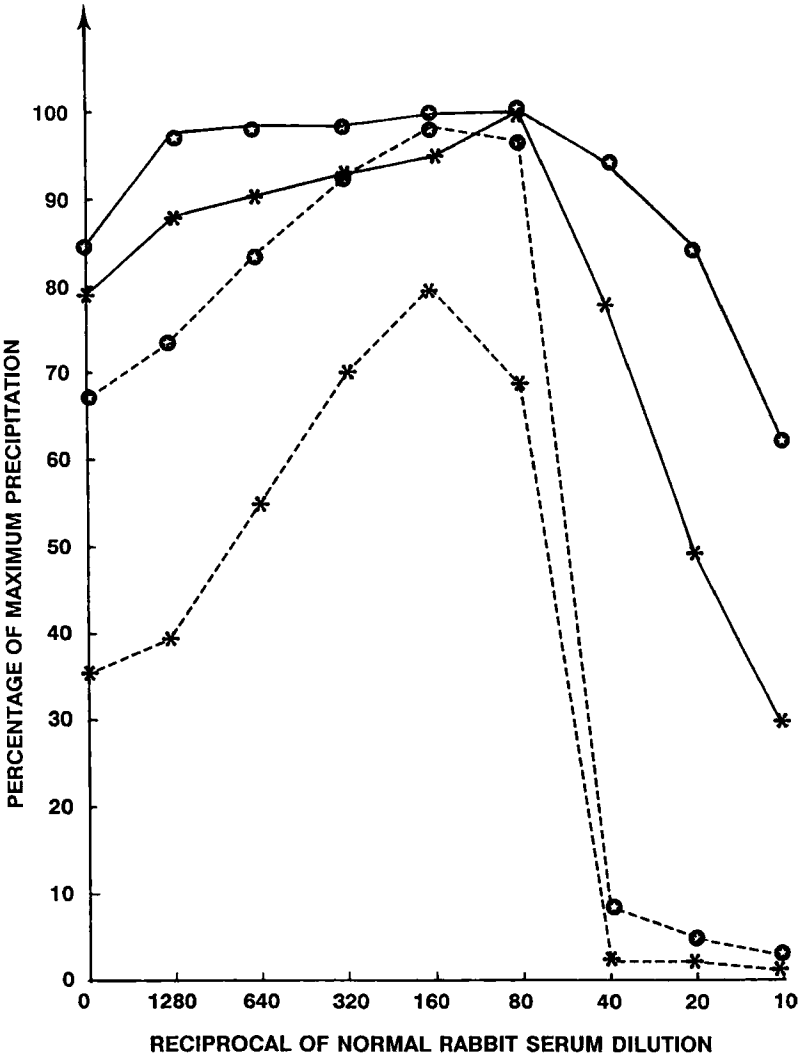

FIGURE 2: Influence of the nature of the antigen on the shape of precipitation curves. Precipitation curves in absence (- - -) or presence (——) of PEG (5 %) in assays for cAMP (✱) and laminin (✪). Goat second antibody diluted 25-fold. 100 % precipitation corresponds to B/T = 53 and 45 % respectively (non specific binding < 5 %).

presence of PEG, it appeared that the nature of the antigen could influence immunoprecipitation; we obtained more effective broadening of the curves with PEG in the case of high molecular weight antigens and this was more pronounced in the excess antigen (as first antibody or carrier immunoglobulins) part of the curves. As an example, Fig. 2 illustrates the curves obtained in two systems: cAMP and laminin using the same goat anti-rabbit IgG antibody. Moreover, as in substance P assay (Fig. 1), we observed an increase of maximum precipitation in cAMP assay, but not in laminin assay (Fig. 2). In addition, we observed differences in the non-specific binding values which increased in the case of high molecular weight antigens at PEG concentrations of 5 % (Table 2) or higher. This phenomenon is due to non-specific precipitation of antigen induced by PEG itself since it was also observed when the second antibody was replaced by the corresponding non-immune serum. In practice, this PEG-induced precipitation of antigens limits the use of PEG in the case of high molecular weight antigens. This point is discussed below more in details.

Influence of the Nature of PEG

All the experiments described above (Fig. 1 and 2, Table 2) were performed with PEG 6000 (mean Mr: 5000-7000) which is the most commonly used PEG as a separatory reagent in analytical immunology. In order to select the polymer giving optimal results, we tested PEGs with various average molecular weights (200, 600, 1500, 4000, 10000).

TABLE 2

Influence of the nature of the antigen on the non specific binding (in percentage of total activity added) observed in different assays in absence and presence of 5 % PEG (normal rabbit serum and second antibody diluted 160- and 25-fold respectively)

PEG concentration (% w/v)	Assay					
	cAMP	DHECT	PRL	Type IV collagen	Laminin	AChE
0	2	4	2	2	3	12
5	2	5	2	4	7	18

TABLE 3

Influence of the nature of PEG (mean Mr) on the non-specific binding (expressed in percentage of total activity added) observed in type IV collagen assay in presence of two concentrations of PEG

PEG concentration (% w/v)	Mean Mr of PEG					
	200	600	1500	4000	6000	10000
1.25	2	2	2	2	3	3
10	2	2	2	8	12	16

The results obtained with a large molecule (type IV collagen) at 2.5 % PEG concentration are shown in Fig. 3. It appeared that low molecular weight PEGs are less effective than high molecular weight PEGs in broadening precipitation curves. On the other hand, for the same concentration of PEG they induced lower non-specific binding than PEG 6000 (Table 3). Because of these two contradictory effets, low molecular weight PEGs did not significantly improve separation although it was always possible to obtain equivalent or better results in terms either of immunoprecipitation efficiency or of non-specific binding by monitoring the concentration of PEG 6000.

Effects of the Concentration of Carrier Immunoglobulins

In all the experiments detailed above, high concentrations of carrier immunoglobulins were used in the immunoprecipitation step (0.1 mL of non-immune sera diluted 100- to 1000-fold) in order to obtain macroscopic immunoprecipitates. We also studied the possibility of lowering the immunoglobulin concentration, assuming that the presence of PEG would still allow an effective separation even with the formation of a microscopic lattice. In order to measure and minimize the quantity of first antibody or carrier immunoglobulins introduced in the test, these experiments were performed using ^{125}I-labelled mouse IgG and rabbit anti-mouse IgG second antibody (see Methods) so that immunoprecipitation of immunoglobulins could be monitored directly. These measurements were made as a function of the quantity of the second antibody added.

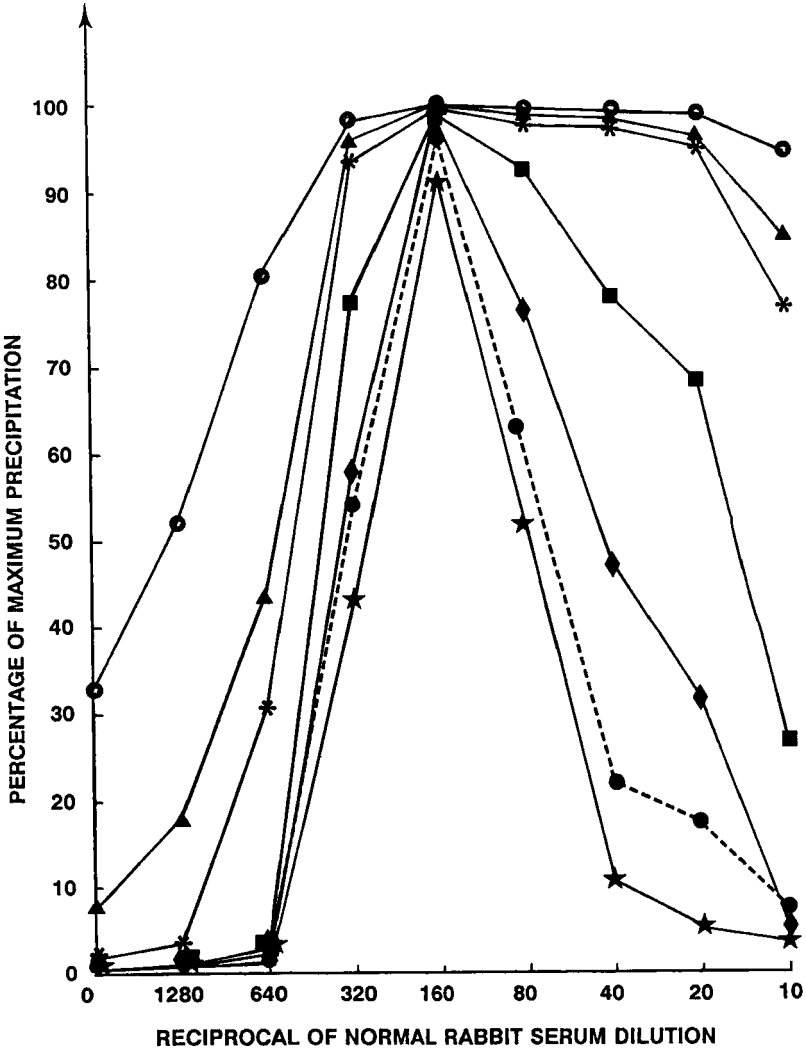

FIGURE 3: Influence of the molecular weight of PEG on the precipitation curves in assay for type IV collagen. The different PEG used were PEG 200 (★), 600 (◆), 1500 (■), 4000 (✱), 6000 (▲) and 10000 (✪) at a 2.5 % final concentration (without PEG: ●). 100 % precipitation corresponds to B/T = 49 % (non specific binding < 5 %).

The results showed (Fig. 4) that, under the conditions used (48 h incubation at room temperature, 2 % PEG, centrifugation 2500 x *g* for 30 min), separation could be achieved with as little as $1:10^4$ diluted immunoglobulins. It is worth noting that for dilutions greater than $1:10^3$ the immunoprecipitate cannot be visualized. As expected, the lowering of the immunoglobulins concentration allowed reduction of the quantity of the second antibody needed. The presence of PEG during immunoprecipitation appeared necessary since complete immunoprecipitation was not observed without PEG for dilutions of immunoglobulins greater than 1:500. However, it is clear that the time required to obtain complete separation was significantly increased with reference to the diminution of the reagents (carrier immunoglobulins, first and second antibodies) concentrations: when 2 h or 6 h reaction periods were chosen (as in other experiments in this study), immunoprecipitation was clearly incomplete when immunoglobulins were diluted more than 500-fold and needed greater incubation time to be complete (data not shown). This is why the curves presented in Fig. 4 refer to 48 h incubation periods. This clearly demonstrates that in any given assay a complete immunoprecipitation could be obtained in presence of PEG without carrier IgG depending on the titer of the first antibody used.

Influence of PEG on the Kinetics of Second Antibody Immunoprecipitation

After having examined the effect of PEG on the precipitation curves under equilibrium conditions, we subsequently studied the effects of PEG on

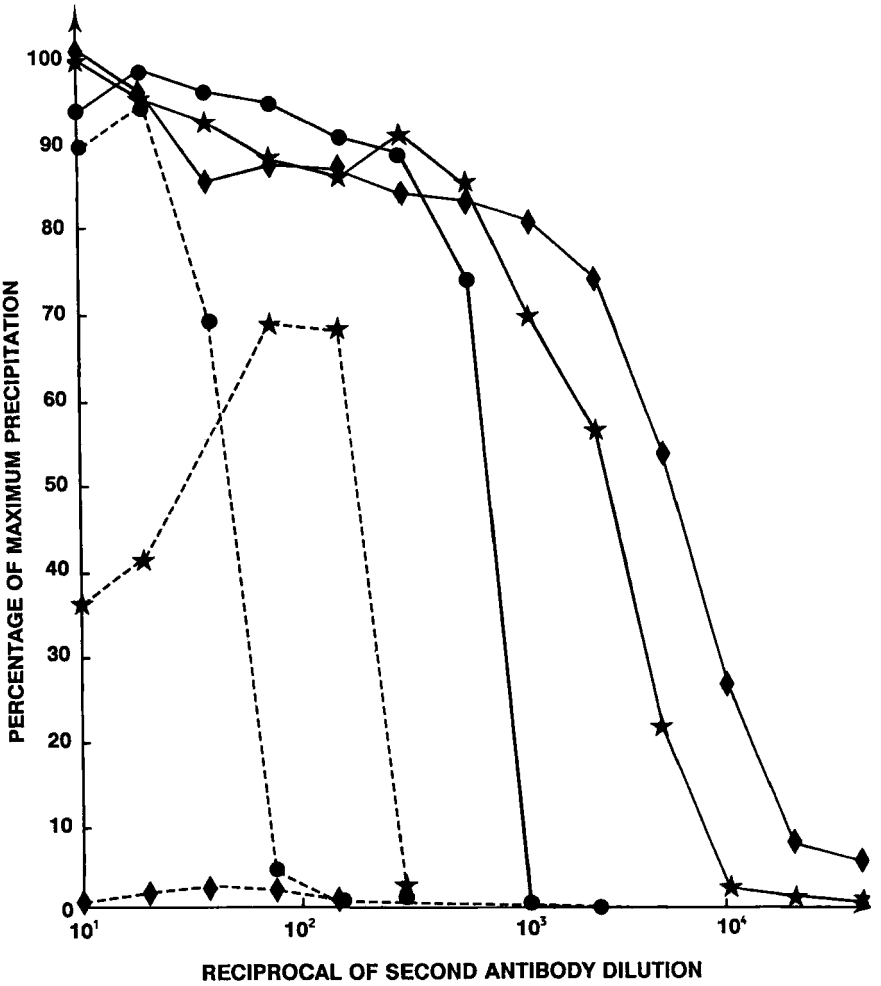

FIGURE 4: Effects of the concentration of carrier immunoglobulins. Precipitation curves in absence (- -) or presence (—) of PEG (2 %) in mouse IgG assay at three different dilutions of labelled mouse immunoglobulins (10^2-, 10^3-, 10^4-fold).● ★ ♦

the kinetics of the second antibody immunoprecipitation. The data obtained for different PEG 6000 concentrations using two systems (substance P and AChE) and the selected goat anti-rabbit IgG serum (see Methods) are shown in Fig. 5A and 5B respectively. In both cases, it appears clearly that the presence of increasing concentrations of PEG accelerated immuno-precipitation. For instance, in the substance P system (Fig. 5A), nearly complete precipitation could be obtained in less than 15 min with 5 % PEG whereas 4 h were necessary without PEG. This was noted regardless of the nature of the antigen, and of the animal species in which the first and second antibodies were raised. Similar but reduced enhancement was obtained with PEGs of lower molecular weight. Moreover, as expected, the precipitation rate decreased when the reaction was performed at lower temperatures and when greater dilutions of the first (or non-immune serum) and second antibody were used (data not shown).

DISCUSSION

During this study we observed two types of effects of PEG on the precipitation of the antigen-antibody complex by the second antibody. First, PEG appeared to influence the immunoprecipitation process under equilibrium conditions in two ways:

- by enlarging the equivalence zone of reaction between first and second antibodies,

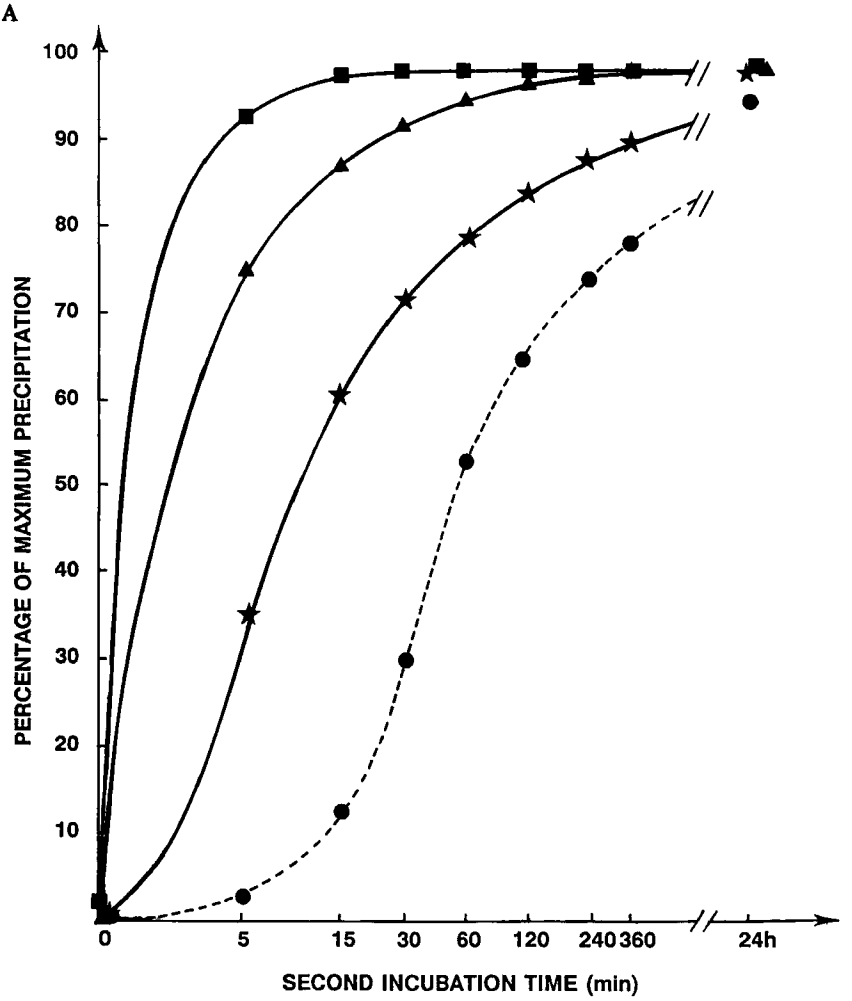

FIGURE 5: Influence of PEG on the rate of second antibody immunoprecipitation. Kinetic studies of the second antibody (goat anti-rabbit antibody diluted 2-fold) immunoprecipitation in absence (●) or presence of 1 % (★), 2.5 % (▲) or 5 % (■) PEG in assays for substance P (5A) and AChE (5B). Normal rabbit serum diluted 200-fold. 100 % precipitation corresponds to B/T = 49 and 72 % respectively (non specific binding < 3 and 14 % respectively).

B

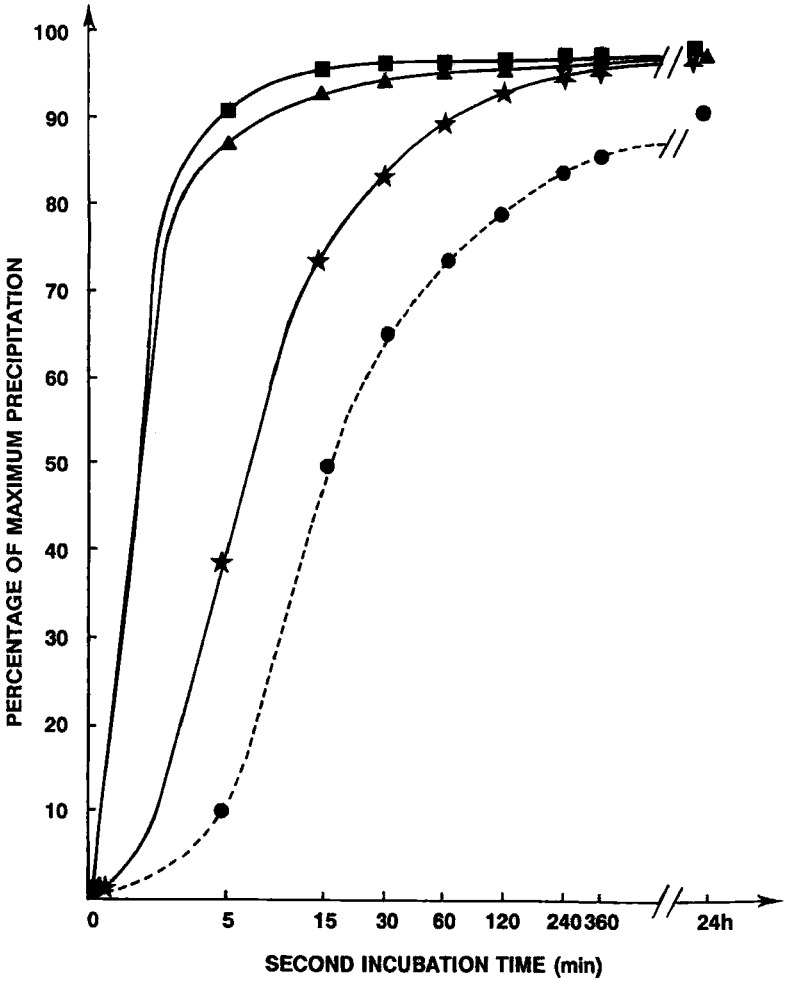

FIGURE 5 Continued

- by greatly accelerating the precipitation of first antibody-second antibody complex.

We observed these two effects qualitatively in all experiments whatever the type of PEG, the antigen, and the first or second antibody used, but quantitative differences were noted upon alteration of any of these parameters. No significant influence of the species producing either antibody could be demonstrated. Moreover, we observed no special advantage in using another PEG than PEG 6000; since there were no great differences between PEGs 4000 and 10000, the heterogeneity of the Mr of PEG 6000 does not appear to be a limitation for its use in these conditions.

The size (i.e. molecular weight) of the molecule involved in the first immune reaction had a significant effect on immunoprecipitation. In the case of macromolecular antigens, PEG appeared more effective in broadening the equivalence zone. This behaviour can be linked to the polyvalence of these large molecules promoting the formation of macromolecular lattices of intrinsically lower solubility which are thus more easily influenced by the presence of PEG (12). On the other hand, and as expected, non-specific binding of these antigens was more critically affected when high concentrations of PEG were used. This phenomenon is not an actual limitation for the use of PEG since significant improvement of the separation method was observed even with low concentrations of PEG (< 2 %) for which non-specific binding remained acceptable (< 15 %) for all the antigens studied (see Table 2). Moreover, an increase of maximum precipitation with

PEG could be observed in several assays of low molecular weight antigens but this was not directly linked to the size of the antigen as it was observed in substance P and cAMP assays (Fig. 1 and 2) but not in DHECT nor in insulin assays. This increase is likely to be associated with the characteristics of both first and second antibodies chosen in the assay (unpublished experiments).

The second type of effect of PEG on second antibody immunoprecipitation resulted in significant increases in the reaction rate. This effect was dependent on PEG concentration and allowed significant reduction in the time devoted to the separation procedure.

Another very interesting feature of PEG is that complete separation is possible even when a microscopic lattice is formed (i.e. when small quantities of both first and second antibodies are used); this enables the quantity of second antibody to be reduced. This attractive possibility, however, is counterbalanced by a significant reduction in the immunoprecipitation rate. One way to circumvent this negative effect would be to introduce the second antibody and PEG together with the first antibody and tracer so that immunoprecipitation occurs at the same time as the first specific immunoreaction. This solution has been adopted sucessfully by some workers (13). However, this approach cannot be generalized since the specific immunoreaction must be independant of both PEG and the second antibody. In addition, the separation conditions (quantities of second antibody, concentration of PEG) must be reevaluated for each immunoassay as a function of the titer of the specific antiserum. As a consequence, this type of

procedure has to be reserved for routine laboratory assays. Another way to use second antibody immunoprecipitation is to work with a fixed concentration of carrier immunoglobulins greater than that of the specific antibody. Under these conditions it is possible to standardize separation conditions, which can be easily adapted to any individual immunoassay. The variable will be the choice of PEG concentration. Clearly, this choice must be a compromise between the positive effects of PEG (broadening of precipitation curve, acceleration of immunoprecipitation) and the negative effects (increase in viscosity, possible increase in non-specific binding).

In practice we would recommend the following procedure:

1) Determination of the optimal amount of second antibody giving maximal precipitation of a predetermined concentration of carrier IgG (usually a 1:50 to 1:1000 dilution of non-immune serum) in the absence of PEG.

2) Measurement of non-specific binding values and reaction kinetics as a function of PEG concentration, other parameters (concentrations of carrier IgG and second antibody, temperature) being predetermined.

3) Choice of the highest PEG concentration compatible with acceptable non-specific binding and viscosity.

Step one is common for all immunoassays involving first antibodies of the same species; it must only be reconsidered when a new batch of second antibody is used. Ideally, steps 2 and 3 have to be reconsidered for each immunoassay. However our experience is that a final PEG 6000 concentration

of 1 or 2 % is suitable for most assays. It should be noted that in the case of haptens for which non-specific binding is not affected by PEG, or in the case of "rapid" second antibody, the choice of the optimal PEG concentration will not be critical since higher concentrations can be used without disadvantages, and separation is possible in a short period even with low concentrations of PEG.

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