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Kinetics of exchange processes in the adsorption of proteins on solid surfaces

(homomolecular exchange)

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ABSTRACT **The homogeneous exchange process whereby IgG molecules adsorbed onto latex particles are replaced by IgG molecules from the bulk solution was studied by means of ^{125}I radiolabeling. The exchange mechanism was investigated on surfaces saturated with either labeled or unlabeled proteins in the presence of a solution of the opposite species in two sets of independent experiments. After rinsing of the surface by pure buffer followed by supplementary IgG adsorption, the exchange process followed a kinetic law of first order with respect to the IgG molecules from the bulk solution, and the apparent exchange rate constant was $(2.3 \pm 0.4) \times 10^{-5}$ cm·hr^{-1}.**

One of the main differences between adsorption processes of "small" molecules and of macromolecules on solid surfaces is that macromolecules can reach the surface by two different mechanisms (1): by direct adsorption or by a progressive exchange reaction. In the first case the molecules reach the adsorption surface directly on free available space. In contrast, for the exchange mechanism the molecules reach the surface by progressive removal of already-adsorbed macromolecules. This occurs in particular when the surface is saturated with preadsorbed molecules. This second process has never been observed in the adsorption of small molecules on surfaces.

The physical origin of this second adsorption process lies in the fact that macromolecules interact with a surface through several links. These links along the macromolecule change with time, even if the molecule is in an equilibrium configuration. Interactions between macromolecule and surface must thus be considered as a dynamic and not a static process. Moreover, macromolecules are not rigid bodies. Thus, if such a molecule enters into the vicinity of a surface already covered by adsorbed macromolecules, it can change its conformation and diffuse into the adsorbed layer. Once part of the backbone of the diffusing molecule reaches the adsorption surface, it interacts with it. This interaction can then be followed by sequential, segmental, reptation-like adsorption of the molecule, in competition with the dynamic process of the formation and annihilation of links of previously adsorbed macromolecules. Finally, the diffusing molecules progressively replace those already adsorbed on the surface, completing the exchange process.

Such processes have been observed for synthetic polymers (2) as well as for proteins (3, 4) by means of radioactive labeling techniques. Nevertheless, and in spite of a great number of investigations, little is known about adsorption processes of macromolecules (5). Let us rapidly summarize the main results that have been established. (*i*) The affinity of proteins for a given surface usually increases with their molecular weight. High molecular weight proteins usually exchange preferentially with lower molecular weight adsorbed proteins (6, 7). (*ii*) The higher the hydrophobicity of the surface is, the larger is the adsorbed quantity (8, 9). (*iii*) These amounts are maximal for pH values close to the isoelectric point of the protein-coated surface (10, 11). (*iv*) For synthetic homopolymers of the same chemical nature, the affinity for a surface increases with the molecular weight (12). (*v*) It has been shown experimentally that for homopolymers of the same nature and the same molecular weight, the exchange process can be modeled by a first-order chemical reaction with respect to both the bulk molecules and the adsorbed polymers (2). This result has been explained theoretically by De Gennes (13). However, it has never been extended to other situations—for example, a mixture of two different molecular weight polymers of the same nature. For proteins, on the other hand, the order of the exchange reaction has never been determined for any situation, despite the importance of this mechanism in the adsorption of proteins onto biosurfaces.

It is the goal of this article to present initial experimental results devoted to this problem. We will not only determine the order of the exchange process but also give an evaluation of the exchange rate constant. The experiments were performed for a system where human IgG molecules in the bulk were in contact with human IgG molecules adsorbed on polystyrene latex particles. This type of protein has been chosen mainly for its importance in biological and pharmaceutical fields—for example, in immunological tests.

MATERIALS AND METHODS

Adsorbents and Adsorbates. Adsorption was onto monodisperse polystyrene latex particles prepared under emulsifier-free conditions (14) using potassium persulfate as initiator. Latex particle size was measured by transmission electron microscopy with Hitachi Ha 12A equipment. From sizing of about 100 particles at the Centre de Microscopie Appliquée á la Biologie (University Claude Bernard, Lyon, France), a number-average diameter (D_n) of 790 nm was calculated with a polydispersity index of 1.001. The specific area was deduced to be 7.3 m^2·g^{-1}. The surface charge of the particles originates from the initiator residues ($-OSO_3^-$) anchored at the water/polymer interface. After the latex particles were cleaned by ionic exchange in mixed-bed resins (Duolite), the amount of covalently bound surface groups was determined by following the conductivity upon titration with

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0.02 M NaOH; the deduced surface charge density was -0.68 $\mu C \cdot cm^{-2}$. The dry weight content of the particles in the initial suspension (pH 7.9) was 9.1%. No flocculation occurred when the latex particles were suspended in the Tris/HCl buffer used throughout this study (50 mM Tris, pH 7.35/1 mM NaCl/1 mM NaN_3).

The proteins employed were polyclonal IgG (weight-average molecular weight ≈150,000) isolated from human serum of about 1000 donors. These proteins were provided by the Centre Régional de Transfusion Sanguine (Strasbourg, France). They were characterized by SDS/PAGE with Coomassie blue staining; purity was judged to be at least 95%.

The IgG molecules were radiolabeled according to the method of McFarlane (15), in which iodine monochloride is the iodinating agent. The amount of NaI was chosen so that about 1 out of 3,000 IgG molecules was labeled. All protein solutions were then stored in concentrated form [$\approx 20 \times 10^{-2}$% (wt/wt)] at −20°C. Before use, they were quickly thawed and diluted to their final concentrations in Tris/HCl buffer. The radioactivities of the labeled solutions were determined by γ counting (Minimaxi γ, United Technologies, Packard). From comparison of the radioactivity with the absorbance at 280 nm [$\varepsilon = 1.38$ $cm^2 \cdot mg^{-1}$ (16)] as measured by a spectrophotometer (Beckman, model 34), the specific activity was determined.

Experimental Procedure. All the experiments were performed in 2-ml Eppendorf tubes. The initial suspension of latex particles was diluted in filtered and deaerated Tris/HCl buffer, with a dilution factor of ≈15. The precise dilution factor for each individual experiment was determined precisely by weighing. All the experiments were performed at room temperature, 23 ± 1°C.

Direct adsorption experiments. Prior to exchange experiments, the direct adsorption conditions must be determined. First, the absence of particle aggregation in the presence of proteins was verified by optical microscopy. Further, to avoid direct adsorption during the exchange process, saturated surfaces should be used and thus plateau adsorption conditions, in particular both characteristic adsorption times and the bulk protein concentration necessary for saturation, had to be evaluated.

Adsorption kinetics were first assessed: 1 ml of diluted latex particles was mixed with 1 ml of a labeled IgG solution of known concentration [2.3×10^{-2}% (wt/wt)] in an Eppendorf tube. After a given adsorption time t, under gentle rotation of 8–10 rpm (Agitest 34050, Bioblock Scientific, Illkirch, France), the tube was centrifuged at 8000 × g for 12 min and the mean activity was determined from three precisely weighed 200-μl samples of supernatant solution. The activity difference before and after the adsorption process allows the determination of the amount of adsorbed protein as a function of time. Great care was taken to avoid the presence of latex particles in the supernatant after centrifugation. Turbidimetric control (turbidimeter model 800, Engineered Systems & Designs, Newark, DE) showed a loss of <0.02% of the particles during this step. All experiments were duplicated. Fig. 1 shows a typical kinetic curve; equilibrium seems to be reached within <2 hr and the final bulk concentration at equilibrium is $(0.45 \pm 0.05) \times 10^{-2}$% (wt/wt).

To determine the adsorption isotherm, the same kind of experiments as just described were performed, but varying the bulk concentration of the labeled proteins (c_{IgG^*}) instead of the adsorption time t, which was held constant at 3 hr. Again, each experiment was duplicated and Fig. 2 represents the evolution of the quantity of adsorbed proteins as a function of c_{IgG^*}. From this, one can conclude that the adsorption plateau of the isotherm at 0.54 $\mu g \cdot cm^{-2}$ is reached for a bulk concentration of the order of 2×10^{-2}% (wt/wt).

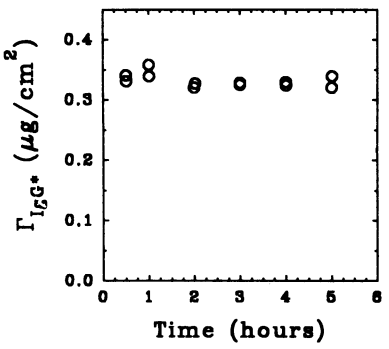

FIG. 1. Adsorption kinetics of labeled IgG, with an initial protein concentration (in the Eppendorf tube) c_{IgG^*} of $(1.5 \pm 0.05) \times 10^{-2}$% (wt/wt), performed at 23 ± 1°C. Each experimental point was duplicated. The final protein bulk concentration in the tube was found to be $(0.45 \pm 0.05) \times 10^{-2}$% (wt/wt).

Moreover, the reproducibility of these experiments can be evaluated to be of the order of 5%.

Exchange experiments. Two types of exchange experiments were performed. (Set I) Kinetic experiments in which the surface of the particles was first saturated with unlabeled IgG molecules, followed by exchange with labeled ones. The bulk concentration of labeled proteins (c_{IgG^*}) was chosen so that its relative variation was <15% over the whole experimental time. For each concentration, six Eppendorf tubes containing latex particles coated with unlabeled proteins were prepared. The amount of adsorbed labeled molecules was then determined after a different reaction time for each tube (in the 1- to 5-hr time interval). This set of experiments was complemented by additional measurements at a constant reaction time of 4 hr. (Set II) Exchange experiments at a constant reaction time t with labeled IgG molecules adsorbed on the particle surface, but in contact with solutions of unlabeled proteins at varying bulk concentrations c_{IgG}.

For all these experiments, IgG molecules were first adsorbed onto the latex particles for 3 hr at an initial bulk concentration of $(3.5 \pm 0.3) \times 10^{-2}$% (wt/wt). This corresponds to adsorption plateau conditions. This step was performed as described in the "direct adsorption" section. After 3 hr of contact between the particles and the proteins, followed by centrifugation, the unadsorbed IgG molecules were removed, avoiding any particle loss. To do this, the latex particles were separated from the solution by centrifugation, and a portion (1.2/2 ml) of the liquid was removed; great care was taken that all latex particles remained in the tubes. A turbidimetric control on the eliminated liquid showed that the loss of particles during this step was <0.5%. The same buffer volume v was then added to the Eppendorf tube and the latex suspension was redispersed. After further

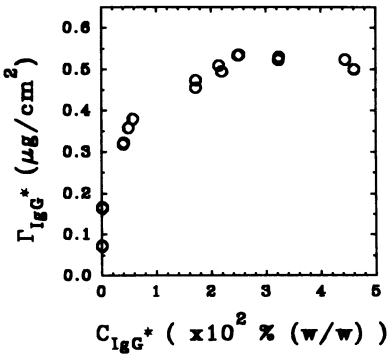

FIG. 2. Adsorption isotherm of labeled IgG at (23 ± 1)°C after 3 hr of adsorption.

centrifugation and redispersion, a control with an optical microscope showed that the particle redispersion was satisfactory (no aggregates were present in the solution). The removal–dilution–redispersion–centrifugation step was repeated four times (five times for the pure desorption experiments, when $c_{IgG} \approx 0$) and led to a dilution ratio of 30–40 (75 for the pure desorption experiments) for the protein solution remaining from the direct adsorption step.

After the last removal–dilution–redispersion–centrifugation step (on average 2 hr after the beginning of the first step), a volume v of protein solution was added to replace the buffer. The protein concentration present in the bulk solution was then c_{IgG} (or c_{IgG^*}). The particles were then redispersed and this was taken as time $t = 0$ for the exchange reaction. This reaction was allowed to take place under a gentle rotation of 8–10 rpm. After a time t, the solution was again centrifuged and the activity of the supernatant was measured.

For the experiments of set I, the difference between the activity of the supernatant after the exchange step and the initial activity of the solution corresponds to the fixed amount of labeled proteins during the exchange step. In set II, the activity of the supernatant was found to be very small. Care must be taken to account for the activity due to any labeled proteins remaining in the solution from the initial adsorption step. Hence, we proceeded as follows: after the direct adsorption step with the labeled proteins, the adsorbed amount Γ_{IgG^*} was determined as described above. Unlabeled IgG molecules were added to the solution (after the four removal–dilution–redispersion–centrifugation steps) to allow for the exchange. At the end of the exchange step and before centrifugation, the activity of the solution (composed by both the latex particles and the exchanging protein solution) was measured. The solution was then centrifuged and the activity of the supernatant determined. The difference between these two quantities corresponds to the amount of labeled proteins Γ^* that remained on the surface of the particles, and the difference between Γ_{IgG^*} (value of direct adsorption at the plateau) and Γ^* represents the amount $\Delta\Gamma_{r^*}$ (where subscript "r" means "released") of proteins that were released from the surface during the exchange step. This method requires the determination of three activities, which leads to more scatter in these experimental results compared with those from experimental set I.

For all the exchange processes taking place in these experiments, the relative variations of the bulk protein concentration and the surface concentration of adsorbed proteins were small (<15%). Consequently, we assumed these concentrations to remain constant for the analysis of the kinetic laws.

RESULTS AND DISCUSSION

We first performed experiment set I. Fig. 3 shows the typical evolution of $\Delta\Gamma_{IgG^*}$, the protein amount that adsorbs on the surface of the particles during the exchange step, as a function of the reaction time t. Two characteristic periods appear on the graph: (*i*) a rapid process taking place during approximatively the first 2 hr (for the experimental conditions under study) followed by (*ii*) a slower and quasilinear increase with time (of slope S_e, the symbol "e" being used for "exchange") of the amount of labeled proteins that adsorb on the surface. Independent experiments were made for 10 different bulk concentrations. The values of S_e were determined from the kinetic curves by a linear least-squares fitting procedure; the linear correlation coefficients were in the range 0.93–0.98. A linear dependence of S_e with bulk concentration c_{IgG^*} was observed (Fig. 4). During the exchange process, the relative variations of c_{IgG^*} were <15%. Moreover, the experiments were realized with IgG* bulk concentrations that were close to or within the adsorption plateau

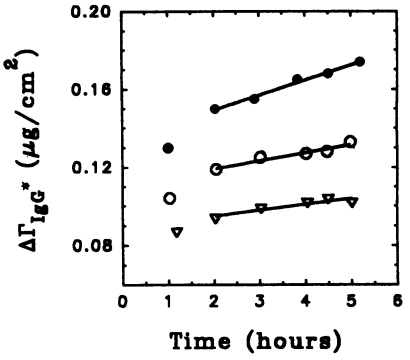

FIG. 3. Examples of kinetic experiments according to set I. $\Delta\Gamma_{IgG^*}$, surface concentration of labeled IgG for various bulk concentrations c_{IgG^*}: ▽, $(1.22 \pm 0.05) \times 10^{-2}$%; ○, $(1.97 \pm 0.05) \times 10^{-2}$%; ●, $(3.60 \pm 0.10) \times 10^{-2}$%. Straight lines correspond to least-squares fits in the interval 2–5 hr.

domain. Due to the large amount of unlabeled adsorbed proteins, the surface concentration of the latter is assumed to be constant. Consequently the linear dependence of S_e with c_{IgG^*} indicates that the process under study can be modeled by a chemical reaction of order 1 for the IgG* molecules in the bulk. Thus, the amount of exchanged proteins $\Delta\Gamma_{IgG^*}$ varies as

$$d(\Delta\Gamma_{IgG^*})/dt = k_e f(\Gamma_{IgG,Tot}) c_{IgG^*}, \quad [1]$$

where $f(\Gamma_{IgG,Tot})$ is an unknown function of the amount of adsorbed protein $\Gamma_{IgG,Tot}$ (considered as constant over the experimental period between 2 and 5 hr), and k_e is the kinetic constant for the exchange. The value of $k_e f(\Gamma_{IgG,Tot})$ can be deduced from Fig. 4, yielding $(2.3 \pm 0.4) \times 10^{-5}$ cm·hr^{-1}.

To prove that the adsorption process described in the experiments of set I is indeed due to an exchange mechanism (and not to further adsorption), it is necessary to demonstrate that—correlated to the adsorption process—proteins are released from the saturated surface of the particles. This was the aim of the experiments of set II lasting over an exchange period of 4 hr, with unlabeled proteins in the bulk solution. The direct preadsorption was performed with labeled proteins. Fig. 5 represents the amount $\Delta\Gamma_{r^*}$ of molecules released from the particles during the exchange mechanism as a function of the bulk concentration c_{IgG} measured at the beginning of the experiment. The experimental data were plotted against the initial and not the final bulk concentration of proteins (as we have done above). Indeed, the final concentration could not be evaluated because the proteins

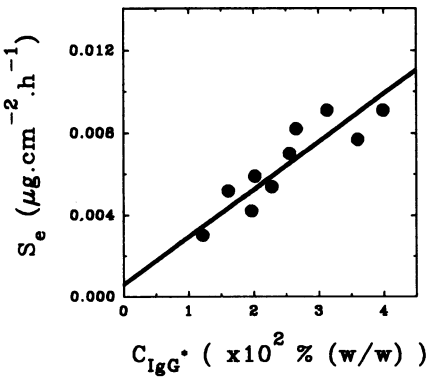

FIG. 4. Slopes (S_e) from Fig. 3 (and the seven experiments not presented), plotted against final bulk concentration c_{IgG^*}. Straight line corresponds to a least-squares fit with a linear correlation coefficient of 0.95. A Student's test showed that the intercept with the y axis was statistically indistinguishable from zero.

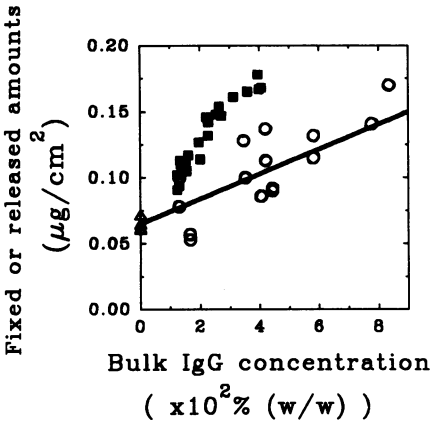

FIG. 5. ○, Released amounts of labeled IgG* ($\Delta\Gamma_{r^*}$) from latex particles (saturated with IgG*) versus c_{IgG} at a constant reaction time of 4 hr. △, Pure desorption experiments ($c_{IgG} \approx 0$). Solid line was calculated from the kinetic law deduced from Fig. 4 with $k_e{\cdot}f(\Gamma_{IgG,Tot}) = (2.3 \pm 0.4) \times 10^{-5}$cm·hr^{-1} and a y intercept of 0.06 μg·cm^2. ■, Fixed amounts of labeled IgG* (Δ_{IgG^*}) versus c_{IgG^*} at the same reaction time. Data include the values from the 10 kinetics measurement (set I) and from 11 additional measurements (see text).

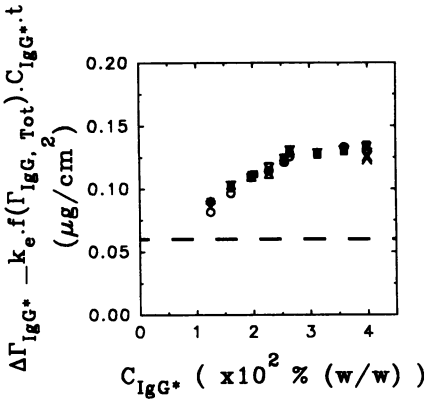

FIG. 6. Calculated values of $\Delta\Gamma_{IgG^*} - k_e{\cdot}f(\Gamma_{IgG,Tot}){\cdot}c_{IgG^*}{\cdot}t$, for the different reaction times with the experimental data from set I, versus c_{IgG^*}. Symbols ○, □, △, ●, and ▽ correspond to the reaction at 2, 3, 4, 4.5, and 5 hr, respectively. Dashed line shows the amount of labeled molecules that replaced the desorbed ones (0.06 μg·cm^{-2}; see Fig. 5). X, Additional experiments performed according to set I with only labeled molecules (see text).

were unlabeled. However, as in set I the relative variations in c_{IgG} should be <15%. A value of about 0.06 μg·cm^{-2} was measured for $\Delta\Gamma_{r^*}$ when the particles were brought in contact with pure buffer ($c_{IgG} = 0$) instead of an unlabeled protein solution. This value corresponds to almost pure desorption, since at the end of the experiment, the concentration of labeled proteins in the buffer, which is due to pure desorption, was $<0.2 \times 10^{-2}$% (wt/wt). The values for $\Delta\Gamma_{r^*}$ increase as c_{IgG} is increased. The large scatter in the experimental values does not allow us to affirm conclusively a linear dependence of $\Delta\Gamma_{r^*}$ on c_{IgG}, but such a dependence is compatible with the observed data.

If one compares the exchange processes performed in the experimental sets I and II, the amount adsorbed (set I) and the amount released (set II) for a given bulk concentration should be identical within the experimental precision. This is not the case, as can be observed in Fig. 5 (difference between ■ and ○). However, if one assumes a linear dependence of $\Delta\Gamma_{r^*}$ with c_{IgG}, with slope equal to its value determined from set I and passing through the value of $\Delta\Gamma_{r^*}$ at $c_{IgG} = 0$ (quantity released by pure desorption), one recovers a good agreement for set II (straight line in Fig. 5) with the experimental data of set I.

To understand the origin of the differences between the adsorbed and released amounts, given by experimental sets I and II, respectively, we reanalyze the data from the kinetic experiments (set I) by focusing our attention on the difference between the amount $\Delta\Gamma_{IgG^*}$ adsorbed during the exchange step and the amount $k_e{\cdot}f(\Gamma_{IgG,Tot}){\cdot}c_{IgG^*}{\cdot}t$ actually attributed to the exchange mechanism. Fig. 6 shows the evolution of $\Delta\Gamma_{IgG^*} - k_e{\cdot}f(\Gamma_{IgG,Tot}){\cdot}c_{IgG^*}{\cdot}t$ as a function of c_{IgG^*}. It can first be noticed that these quantities are almost independent of time and thus depend only on c_{IgG^*}. This again shows that at least two mechanisms with different time scales are involved during the exchange step: the slow exchange process, characterized by a linear dependence with time and with bulk concentration, and a more rapid process whose contribution is plotted in Fig. 6. The curve shows a plateau domain for protein concentrations above (2–2.5) $\times 10^{-2}$% (wt/wt) as for the direct adsorption isotherm (Fig. 2). The observed plateau value in Fig. 6 is close to 0.125 μg·cm^{-2}.

Due to its resemblance to the adsorption isotherm, we suggest that this rapid process is due to additional adsorption. This adsorption occurs after the four removal–dilution–redispersion–centrifugation steps as soon as the surface covered with unlabeled molecules is brought in contact with the labeled ones.

Such a process cannot be observed when unlabeled proteins are introduced after the same dilution steps, as in set II. Only the release of labeled proteins can be observed in that case; this included a pure desorption (independent of the presence of additional proteins, see above) contribution of 0.06 μg·cm^{-2} which should also occur in set I. Some of the additional adsorption after the adsorption–rinse–adsorption cycle would replace this desorbed protein amount, but the higher additional adsorption, with a plateau value of 0.125 μg·cm^{-2} as seen in Fig. 6, suggests, if our assumption is correct, that the plateau value of the isotherm is increased by a supplementary amount of about 0.06 μg·cm^{-2}. This was in fact observed in two additional experiments performed as in set I but by adsorbing labeled IgG both during the initial adsorption period and during the adsorption–exchange step. These two additional experiments were performed with $c_{IgG^*} = (4.0 \pm 0.3) \times 10^{-2}$% (wt/wt) in the plateau region. The total amount of adsorbed material was measured as in set II from the difference between the activity of the homogeneous solution and the supernatant after centrifugation. In the first experiment, the measurement was performed after 4 hr; a supplementary adsorbed amount of 0.06 μg·cm^{-2} was in fact found (Fig. 6). In a second kinetic experiment (Fig. 7) we

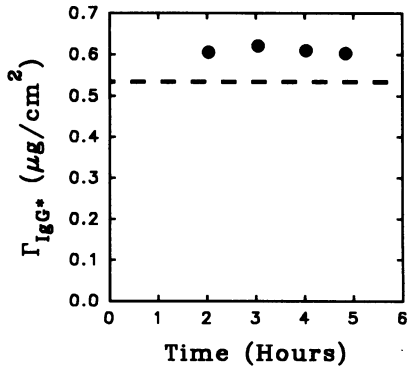

FIG. 7. Kinetics of supplementary adsorption that takes place after the four removal–dilution–redispersion–centrifugation steps. Labeled IgG molecules at an initial concentration of $(3.5 \pm 0.1) \times 10^{-2}$% (wt/wt) were first adsorbed onto the surface of the particles, and after the dilution steps, labeled proteins at $(4.0 \pm 0.3) \times 10^{-2}$% (wt/wt) were added. Dashed line corresponds to the mean adsorbed value after the first adsorption step: 0.54 μg·cm^{-2}.

followed the supplementary adsorption in the interval 2–5 hr. The readsorbed amount remained constant in this time interval, confirming that the further adsorption observed in set I after 2 hr was indeed protein exchange. The additional adsorbed amount (Fig. 6), behaving like the direct adsorption isotherm, could be the consequence of an interfacial rearrangement taking place in the adsorbed layer during the removal–dilution–redispersion–centrifugation steps. If one subtracts from the data of experimental set I (for a reaction time equal to 4 hr; see Fig. 5) this additional adsorbed amount (data from Fig. 6 minus the 0.06 $\mu g \cdot cm^{-2}$ attributed to the replacement of desorbed molecules), one finds a linear variation of the exchanged amount (total amount minus the additional adsorption contribution) with the bulk concentration (Fig. 8). The linear regression by least-squares fit (correlation coefficient, 0.92) intercepts the y axis at 0.045 $\mu g \cdot cm^{-2}$, a value which is close to the one found previously in the pure desorption experiment.

The straight line confirms moreover that the exchange process is of first order with respect to the molecules in the solution. The slope of the straight line must again be equal to $k_e \cdot f(\Gamma_{IgG,Tot}) \cdot t$ (with t = 4 hr). The experimentally deduced value of $(2.9 \pm 0.8) \times 10^{-5}$ $cm \cdot hr^{-1}$ found for $k_e \cdot f(\Gamma_{IgG,Tot})$ is in good agreement with that deduced from the kinetic data (set I), $(2.3 \pm 0.4) \times 10^{-5}$ $cm \cdot hr^{-1}$. Moreover, the fixed and the released amounts involved in the exchange process are equal within experimental precision.

From these sets of experiments one can conclude that we have described the exchange process for the system IgG/IgG on latex particles. We have also demonstrated (and to our knowledge this has never been done before for proteins) that such an exchange process can be modeled by a chemical reaction of order 1 with respect to the bulk molecules, which corresponds to a kinetic law given by Eq. 1. The apparent

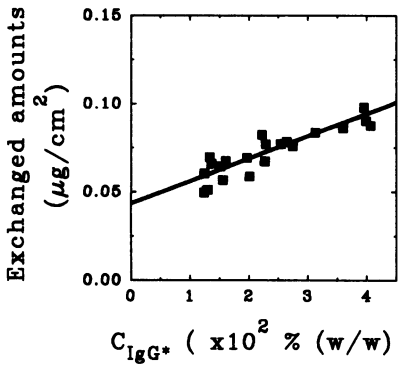

FIG. 8. Amount of IgG* exchanged with the unlabeled IgG-saturated latex particles after 4 hr of reaction, versus c_{IgG^*}, calculated after subtraction of the additional adsorbed quantities (values above 0.06 $\mu g \cdot cm^{-2}$ in Fig. 6) from the data plotted in Fig. 5. Solid line shows least-squares fit for the data of set I at 4 hr of reaction.

reaction rate constant has also been evaluated and is of the order of $(2.3 \pm 0.4) \times 10^{-5}$ $cm \cdot hr^{-1}$. These experiments do not allow us to determine the order of the reaction with respect to the adsorbed protein molecules, as has been done for polymers (2). Such a determination would require labeling only part of the adsorbed proteins and performing similar experiments as in set II. The data in Fig. 5 clearly demonstrate that the accuracy of the results would be insufficient to draw any absolute conclusion about the order of this reaction. However, it is likely that the order of the reaction is equal to 1 with respect to the adsorbed molecules. If one assumes such a second-order law, the kinetic equation for the adsorption process becomes

$$d\Delta\Gamma_e/dt = k_e \cdot \Gamma_{IgG,Tot} \cdot c_{IgG^*}. \quad [2]$$

The kinetic constant k_e can then be estimated at $(5.8 \pm 1.0) \times 10^3$ $liter \cdot mol^{-1} \cdot hr^{-1}$ for a Γ_{IgG} value of 0.60 $\mu g \cdot cm^{-2}$ (we used 0.60 $\mu g \cdot cm^{-2}$ instead of 0.54 $\mu g \cdot cm^{-2}$ since we have shown that the plateau value of the isotherm increases after the four removal–dilution–redispersion–centrifugation steps).

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