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Ligand location and biochemical productivity of silica-based immunoaffinity adsorbents

S. B. Mohan and A. Lyddiatt

Biochemical Recovery Laboratory, School of Chemical Engineering, University of Birmingham, Edgbaston, Birmingham B15 2TT (UK)

ABSTRACT

Ligand location within particles, detected by immunogold labelling, was shown to influence the biochemical productivity of a silica-based solid phase, Sorbsil C-500, using a model ligand–biomolecule system (immobilised human immunoglobulin G–anti-human immunoglobulin G monoclonal antibody). The distribution of the ligand was in turn affected by the initial ligand challenge used to prepare the immunoadsorbents. Maximal productivity was achieved with adsorbents prepared with an initial challenge of about 3 mg human immunoglobulin G per ml: the ligand in these cases was shown to be more uniformally distributed within the adsorbent particles than adsorbents, exhibiting low productivity, prepared with either low (1 mg/ml) or high (9 mg/ml) concentrations of human immunoglobulin G. The ligand in the latter was restricted to the periphery of the particles.

INTRODUCTION

Affinity chromatography exploits the unique specificity inherent in a ligand–biomolecule interaction (as for example in antibody–antigen, receptor–hormone and enzyme–inhibitor interactions) and provides a powerful technique for single-step purification of desired products from crude broths. At present solid phases based on chemically cross-linked agarose (4 or 6%) are used commonly in affinity chromatography [1–3]. However, agarose lacks rigidity and to address this many silica-based and synthetically assembled matrices have been promoted to replace agarose. Agarose is a macroporous solid phase having 40–160 μm diameter beads, 100–300 nm pores and a molecular size exclusion of $40 \cdot 10^6$ kDa [4]. In contrast, many commercial silica supports have pores in the mesoporous range (8–30 nm diameter) and, though sold as wide-pore solid phases, their physical characteristics clearly conflict with the estimated physical diameters of proteins commonly exploited as ligands and products (10–150 kDa, 2–10 nm diameter [5]). A number of studies have shown that the pore size of a solid phase affects its performance [6,7] but the extent of these effects has been the focus of study only recently [8]. The latter reported the influence of pore size upon the performance of three silica-based solid phases, Sorbsil C-200, C-500 and C-1000 (referred to as C-200, C-500 and C-1000 in the text), having particle diameters of 40–60 μm and corresponding average pore diameters of 20, 50 and 100 nm, using model ligand–biomolecule systems of varying molecular sizes.

Two unique parameters, biochemical productivity (the molar ratio of the amount of product recovered per unit volume of the adsorbent and ligand concentration) and maximum physical capacity (the theoretical maximum physical capacity of the adsorbent to accommodate the biomolecules), were identified as generically essential for optimum efficiency of an affinity adsorbent.

Correspondence to: S. B. Mohan, Biochemical Recovery Laboratory, School of Chemical Engineering, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK.

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Using an immobilised human immunoglobulin G (Hu-IgG) anti-Hu-IgG monoclonal antibody system, in which both the ligand and the product are of the same size, it was shown that the physical capacity of C-200 to accommodate the ligand and the product was only 16% of the theoretically expected amount. This capacity increased to 70 and 90% of the expected value with C-500 and C-1000, respectively. This difference in capacities was attributed to the pore size which in turn affected the distribution of ligand within the adsorbent particles.

Immunolabelling of particle sections by fluorescence and gold staining demonstrated that the ligand was restricted to the peripheral 3 μm of the C-200 particles but was distributed uniformally within the C-1000 particles, suggesting that the latter solid phase allowed free access of biomolecules. The adsorbents used in the above study were prepared with a ligand challenge of about 3 mg Hu-IgG per ml. This report extends the previous study and illustrates the effect of the initial ligand challenge upon the biochemical productivity of these solid phases.

EXPERIMENTAL

Materials

Epoxy silica matrices, Sorbsil C-200, Sorbsil C-500 and Sorbsil C-1000 (designated C-200, C-500 and C-1000, respectively), were supplied by Crosfield Chemicals (Warrington, UK). The particles were 40–60 μm in size with pore diameters of 20, 50 and 100 nm, respectively, as defined by size exclusion chromatography of polystyrenes [9]. All biochemicals were obtained from Sigma unless stated otherwise.

Continous culture of TB/C3 hybridoma cells for the production of monoclonal antibodies

Murine hybridoma cells (TB/C3), cloned by Dr. B. Williams from a WC2 cell line donated by Dr. R. Jefferies, Department of Immunology, University of Birmingham, were the kind gift of Dr. A. N. Emery, Mammalian Cell Culture Group, School of Chemical Engineering, University of Birmingham. The cells, which produce monoclonal antibodies (MCAB) to $C\gamma_2$ domain of Hu-IgG, were grown in continuous culture as described previously [10]. MCAB from the spent medium was purified by affinity chromatography on Hu-IgG–Sepharose CL-4B (25 ml bed volume) as described below and stored at −20°C until required.

Purification of Hu-IgG

Hu-IgG was purified from outdated blood obtained from the National Blood Transfusion Centre (Birmingham, UK). Plasma was collected by centrifugation (5000 *g* for 10 min at room temperature) and 50 ml, diluted ten-fold with 10 m*M* Tris buffer, pH 7.5, was applied to ZETAPREP QAE (Pharmacia) previously equilibrated in the above buffer. Breakthrough fraction of Hu-IgG was collected and concentrated by precipitation with ammonium sulphate (40% saturation). The precipitated protein was collected by centrifugation (20 000 *g* for 20 min at 4°C), dissolved and dialysed against phosphate-buffered saline (PBS: 0.02 *M* potassium phosphate, pH 7.4, containing 0.15 *M* sodium chloride and 15 m*M* sodium azide) and stored at −20°C until required.

Preparation of immunoadsorbents

Sepharose CL-4B was activated by cyanogen bromide and coupled to Hu-IgG [11]. Unbound ligand was removed by washing the adsorbent sequentially with ten volumes each of sodium bicarbonate buffer (0.1 *M*, pH 9.0, containing 0.5 *M* sodium chloride), sodium acetate buffer (0.1 *M*, pH 4.0, containing 1.0 *M* sodium chloride) and bicarbonate buffer. Remaining reactive sites were blocked by washing the matrix with ten volumes of 0.3 *M* ethanolamine, pH 8.0, followed by end-over mixing for 2 h at room temperature in five volumes of the blocking reagent. The adsorbent was equilibrated in PBS and stored at 4°C until required. An adsorbent blank prepared simultaneously (without Hu-IgG in the coupling buffer) was used to monitor the success of the blocking step: successfully blocked preparation did not adsorb any proteins present in the test samples.

Epoxy C-200, C-500 and C-1000 silicas were hydrolysed to diol forms by stirring 10 g of ma-

trix suspended in 500 ml of 0.1 *M* hydrochloric acid at 90°C for 4 h. The diol matrices were washed in water, transferred to dioxane and activated by 1,1′-carbonyldiimidazole (CDI; 10%, w/w [12]) for 30 min. Activated material was transferred to water and suspended in PBS containing Hu-IgG in appropriate concentrations. Suspensions were agitated at room temperature for 2–4 h. Unbound ligand was removed by washing sequentially with PBS, PBS containing 1 *M* sodium chloride and PBS. Remaining reactive sites were blocked and the adsorbents equilibrated as described above.

In all cases, the concentration of the bound ligand (*L*) was determined by the difference in protein concentration between the starting solution and the solution obtained after the washing step to remove the unbound ligand. An adsorbance at 280 nm of 1.4 for 1 mg/ml Hu-IgG was used to determine the protein concentration [13].

Affinity chromatography

MCAB was purified on a fixed bed of Sepharose CL-4B–Hu-IgG (2.0 × 10 cm), previously equilibrated in PBS. Clarified culture medium was applied and the bed washed with PBS until no protein was detected in the eluate, followed by ten volumes each of PBS containing 1 *M* sodium chloride (to remove any non-specifically adsorbed protein) and PBS. Adsorbed protein was eluted with 3 *M* KSCN and desalted immediately on a Sephadex G25 bed (2 × 30 cm) connected downstream of the affinity adsorbent.

Remaining adsorbents were packed into 1–2 ml beds and equilibrated with PBS. Purified MCAB was applied and the beds developed as described above except that the desorbed proteins were desalted on 1 × 10 cm beds.

Productivity of the solid phases in fixed beds

Beds of 1 ml were saturated by applying an excess of purified MCAB by recycling for 16 h, at 4°C. The beds were washed and developed as described above to obtain the amount of product recovered (*R*) from the matrix and hence its productivity (*P*, the molar ratio of *R* and the ligand concentration, *L*, per ml adsorbent).

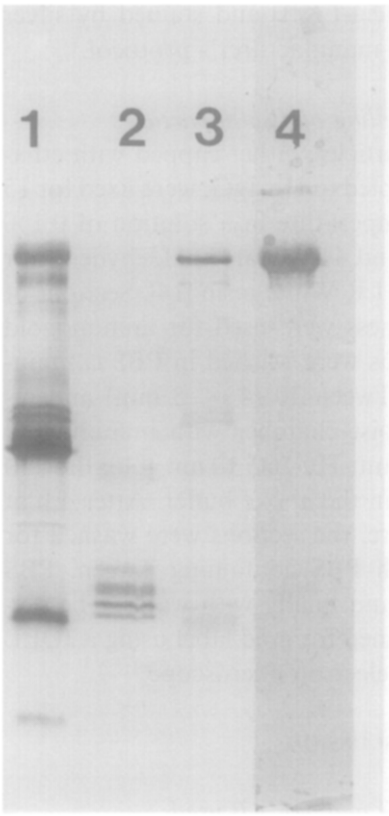

Fig. 1. SDS-PAGE, under unreduced conditions, of Hu-IgG and MCAB purified from plasma and cell culture medium, respectively. Lanes 1, 2 and 3 contained crude cell culture medium, non-specific proteins desorbed with PBS containing 1 *M* NaCl and purified MCAB, respectively; lane 4, purified Hu-IgG.

Maximum physical capacity of the solid phases

The pore volume available for binding was similar in the three solid phases studied (37% of the total particle volume; Dr. I. Chappell, Crosfield Chemicals, personal communication). This volume and the Stokes' radii of the proteins studied [5] were used to calculate the theoretical maximum physical capacity of the matrices assuming that there was no steric hindrance to the access of molecules. This theoretical value (18 mg IgG per ml matrix) was used to evaluate the maximum physical capacity actually achieved in fixed beds ($L + R$).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Gradient gels (10–15%) were run on a Phast-

System (Pharmacia-LKB) and stained by silver according to the manufacturer's protocol.

Immunogold labelling of C-500 particles

Diol C-500 particles, either capped with ethanolamine or coupled to Hu-IgG, were fixed for 15 min at room temperature in a solution of 0.1% glutaraldehyde and 4% paraformaldehyde, prior to embedding in LR White resin [14]. Sections of 10–15 nm thickness were used for immunogold labelling. Sections were washed in PBS containing 0.1% (v/v) Tween 20 (4 × 5 min) and incubated in a moist chamber with immunogold conjugate (goat anti-Hu-IgG 10 nm gold; Biocell) diluted ten-fold in the above buffer. After 4 h at room temperature, the sections were washed for 4 × 5 min with PBS containing Tween, PBS without Tween and finally with water. The sections were examined for gold label using a JOEL 100CX temscan electron microscope.

RESULTS AND DISCUSSION

Purity of Hu-IgG and MCAB used

The purity of Hu-IgG and MCAB used to prepare immunoadsorbents and to obtain fixed bed data, respectively, was checked by SDS-PAGE. Fig. 1 (tracks 3 and 4) shows that both the components of the system studied produced predominantly 150 kDa bands in unreduced gels, indicating their high purity.

Effect of initial ligand challenge on the performance of C-200–, C-500– and C-1000–Hu-IgG adsorbents

The three solid phases were challenged with varying concentrations of Hu-IgG to obtain immunoadsorbents with a range of ligand concentrations. The characteristics of these are summarised in Table I. C-200 matrices prepared with initial ligand challenges of 1.3–6.0 mg Hu-IgG per ml, and total Hu-IgG challenges of 3 and 6 mg/ml matrix, yielded three out of four adsorbents having ligand concentrations (L) of 2.0–2.1 mg/ml. The amount of MCAB recovered (R) from fixed beds was also similar in these cases (0.6–0.65 mg/ml matrix) suggesting that the variation in initial or total ligand challenge did not affect the productivity (P, molar ratio of product and ligand concentration) of this solid phase. However the value of P (0.3) was lower than the expected stochiometry of 2. The fourth C-200 matrix had a lower ligand concentration (1.3 mg/ml) but the amount of MCAB recovered from this adsorbent (1.13 mg) and its productivity (0.9) were higher than the rest. This adsorbent was prepared with an initial and a total challenge of 3 mg Hu-IgG per ml; these might, therefore, represent the coupling conditions required for the optimum performance of this matrix.

C-500 challenged with a low initial Hu-IgG concentration (0.9–1.25 mg/ml) reacted with less ligand as the total Hu-IgG challenge per ml matrix increased, the incorporation being 90, 82 and 63%, respectively, for total challenges of 2.1, 3.0 and 9.0 mg (Table I). The corresponding immobilised ligand concentrations achieved were 1.9, 2.45 and 5.7 mg Hu-IgG per ml matrix. The maximum amount of MCAB which could be recovered from saturated beds of these adsorbents was 1.98, 2.36 and 3.4 mg/ml matrix, equivalent to the productivity values of 1.0, 0.96 and 0.6, respectively. In contrast, as the initial Hu-IgG concentration in the coupling reaction was increased to 2.5–3.3 mg/ml, 93–97% of the ligand was immobilised regardless of the total challenge (3–7.5 mg Hu-IgG per ml matrix) used. The resulting adsorbents, with ligand concentrations of 2.9, 4.2 and 5.3 mg/ml, yielded productivities of 2.0, 1.7 and 1.3, respectively; the former two values approached the expected theoretical value. These productivities were considerably higher than those reported above for adsorbents of similar ligand concentrations (1.9 and 2.45 mg Hu-IgG per ml) prepared using a lower initial Hu-IgG challenge. These data suggest that initial ligand concentration used in the coupling reaction might influence the productivity of the C-500 matrices. Comparision of two adsorbents prepared with high total Hu-IgG challenge of 9.0 mg/ml matrix but an initial concentration of 1 or 9 mg/ml showed that ligand incorporation (63 and 82%, respectively), immobilised ligand concentration (5.7 and 7.4 mg/ml matrix) and the

TABLE I

CHARACTERISTICS OF HU-IgG MATRICES

C-200, C-500 and C-1000 solid phases were challenged with varying concentrations of Hu-IgG to obtain immunoadsorbents with a range of ligand concentrations (L). Fixed-bed characteristics were obtained by saturating 1-ml beds with an excess of purified MCAB. Unbound MCAB was removed by washing the beds with PBS followed by PBS containing 1 M NaCl and PBS. Adsorbed MCAB was eluted with 3 M KSCN and desalted immediately on Sephadex G25 (1 × 10 ml). The amount of MCAB recovered (R) was used to calculate the productivity (P, the molar ratio of R/L). The asterisks indicate adsorbents examined for ligand location.

Matrix	Hu-IgG challenge		L (mg/ml matrix)	Percentage initial challenge reacted	R (mg/ml matrix)	P (R/L)	Physical capacity	
	mg/ml	Total mg/ml matrix					$R+L$	Percentage expected value
C-200	1.3	3.0	2.1	70	0.6	0.3	2.7	15
	1.3	6.0	2.0	33	0.6	0.3	2.6	14
	3.0	3.0	1.3	43	1.13	0.9	2.4	13
	6.0	6.0	2.1	35	0.65	0.3	2.75	16
C-500	0.9	2.1	1.9	90	1.98	1.0	3.88	21
	1.25	3.0	2.45	82	2.36	0.96	4.81	27
	2.5	3.0	2.9	94	5.8	2.0	8.7	48
	2.5	4.5	4.2	93	7.5	1.7	11.7	65
	3.3	5.4	5.2	97	6.7	1.3	11.9	66
	3.3	7.5	7.2*	96	6.2	0.86	13.4	74
	1.0	9.0	5.7*	63	3.4	0.6	9.1	50
	9.0	9.0	7.4*	82	5.1	0.69	12.5	69
C-1000	1.65	1.9	1.8	95	3.4	1.9	5.2	29
	2.5	4.5	4.0	89	7.3	1.8	11.3	63
	3.3	5.4	5.3	98	9.7	1.8	15.0	83
	3.3	8.5	8.3	97	7.8	0.94	16.1	89
	1.0	9.0	9.0	100	9.1	1.0	18.1	101
	9.0	9.0	9.0	100	9.3	1.0	18.3	101

amount of MCAB recovered (3.4 and 5.1 mg/ml matrix) varied in these, indicating that total ligand challenge might also influence the final performance characteristics of C-500 immunoadsorbents. Furthermore, one adsorbent with an immobilised ligand concentration of 7.2 mg/ml, prepared with an initial Hu-IgG concentration of 3.3 mg/ml, showed higher productivity than an adsorbent of similar ligand concentration (7.4 mg Hu–IgG per ml matrix) prepared with 9 mg Hu-IgG per ml. These observations suggest that there was a further parameter, an optimum initial concentration of ligand, which required consideration for an optimal performance of a solid phase. For C-500 this concentration was about 3 mg/ml for the ligand studied.

The data obtained for C-1000 with adsorbents prepared with a range of initial Hu-IgG concentrations (1, 1.65, 3.3 and 9.0 mg/ml) and total ligand challenges (1.9, 4.5, 5.4, 8.5 and 9.0 mg/ml matrix) showed that most of the ligand was coupled to the solid phase in all cases (Table I). Productivity approached the theoretical maximum expected with immobilised ligand concentrations of 1.8, 4.0 and 5.3 mg Hu-IgG per ml matrix regardless of the variations in the initial and total ligand challenges. In contrast to C-500, C-1000 did not show any effect of initial or total ligand challenge. Three adsorbents with high ligand concentrations (8.3, 9.0 and 9.0 mg Hu-IgG per ml matrix) prepared using similar total challenge (8.5–9.0 mg/ml matrix) but varying initial con-

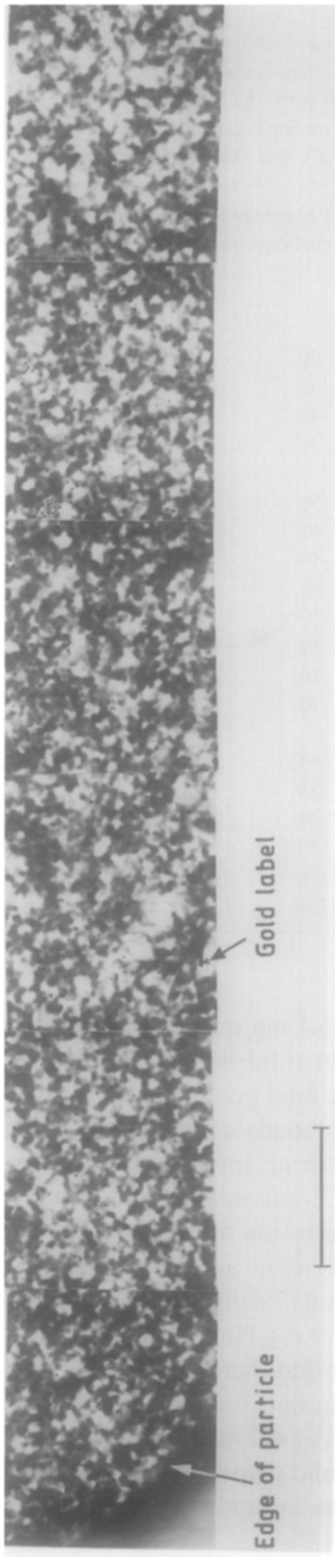

Fig. 2. Distribution of ligand within C-500 immunoadsorbent prepared with an initial challenge of 1 mg Hu-IgG per ml. Thin sections (10–15 nm thick) were stained by immunogold labelling and electron micrographs showing the distribution of gold label from the edge of one particle were used to obtain the composite photograph shown. Bar marker represents 500 nm.

centrations (1.0, 3.3 and 9.0 mg Hu-IgG per ml) showed similar performance characteristics.

The maximum physical capacities ($R + L$) of 2.7, 12–13 and 18 mg/ml 150-kDa protein per ml matrix obtained for C-200, C-500 and C-1000, respectively, corresponding to 16, 70 and 100% of the theoretical expected capacities, are similar to those reported previously [8]. Additional parameters, utilising variations in ligand challenge (initial concentration or total ligand challenge per ml matrix), tested in the present study did not appear to affect the physical capacities available for interactions between the ligand and product in C-200 and C-1000 solid phases. The low capacity of C-200 was shown previously to be due to limited penetration of the ligand (to the peripheral 3 μm of the particles [8]) in an adsorbent with a ligand concentration of 3 mg Hu-IgG per ml. Further increase in capacity was perhaps not feasible because of the steric constraints imposed by the pores of this solid phase. The near theoretical capacity achieved for C-1000 immunoadsorbents and the similar performance of the adsorbents with high ligand concentrations, despite the variables introduced in their preparation, provided evidence for negligible hindrance to access due to the geometry of this solid phase. The binding sites of the immobilised ligand were accessible to the product even at high ligand concentrations when up to 50% of the available physical capacity was utilised. These data substantiate the previous study [8] which showed a uniform distribution of ligand in a C-1000 adsorbent with a ligand concentration of 6 mg Hu-IgG per ml matrix.

In the case of C-500, some influence on the maximum matrix capacity of the variables tested was apparent. Hence, adsorbents with similar immobilised ligand concentrations (5.2 and 5.7 mg Hu-IgG per ml matrix) prepared with varying initial Hu-IgG challenge (3.3 and 1.0 mg/ml) exhibited maximum physical capacities of 66 and 50%, respectively, of the expected value. Moreover, adsorbents with immobilised ligand concentrations of 7.2 and 7.2 mg/ml, prepared using initial Hu-IgG concentrations of 3.3 and 9.0 mg/ml, respectively, showed variation in productivity (0.86 and 0.69) despite exhibiting similar capacities (74 and 69% of the expected value). These data suggest that the parameters determining the physical capacities of these adsorbents might be different.

Effect of initial ligand challenge on the distribution of ligand within C-500 particles

Ligand distribution was shown to account for the productivity of C-200 and C-1000 adsorbents [8]. Immunofluorescent and immunogold staining of sections of C-200– and C-1000–Hu-IgG showed that the ligand was restricted to the peripheral 3 μm of the C-200 particles but was present throughout the C-1000 particles. It was feasible that the performance characteristics documented for C-500 might also be related to ligand location. To determine whether initial and total ligand challenge influenced the distribution of ligand within C-500 immunoadsorbent particles, ligand was located in sections of three adsorbents by immunogold labelling. The adsorbents used (asterisk in Table I) were prepared with a different initial Hu-IgG concentration (1, 3.3 and 9.0 mg/ml) but with similar total ligand challenge (9, 7.5 and 9 mg/ml matrix, respectively). Gold label was undetectable in the control capped matrices to which no Hu-IgG was coupled. Gold label, and hence the ligand was restricted to the peripheral 4.5 μm of the particles of the adsorbent prepared with an initial challenge of 1 mg/ml (Fig. 2). Many gold particles were present in the vicinity of the edge (not shown) suggesting that the ligand probably saturated the binding sites present on the edge of the particle. If so, the penetration of the ligand within the particle would be limited and this would explain the low ligand uptake (63%, Table I) of this adsorbent. It follows therefore that the access of the product would also be restricted and would account for the low MCAB recovery and productivity (3.4 mg MCAB per ml matrix and 0.6, respectively) obtained for this adsorbent.

Gold label was present throughout the particles of the adsorbent prepared with an initial Hu-IgG concentration of 3.3 mg/ml though the density of the gold label was higher at the edge of the particle than that detected at a depth of 14 μm

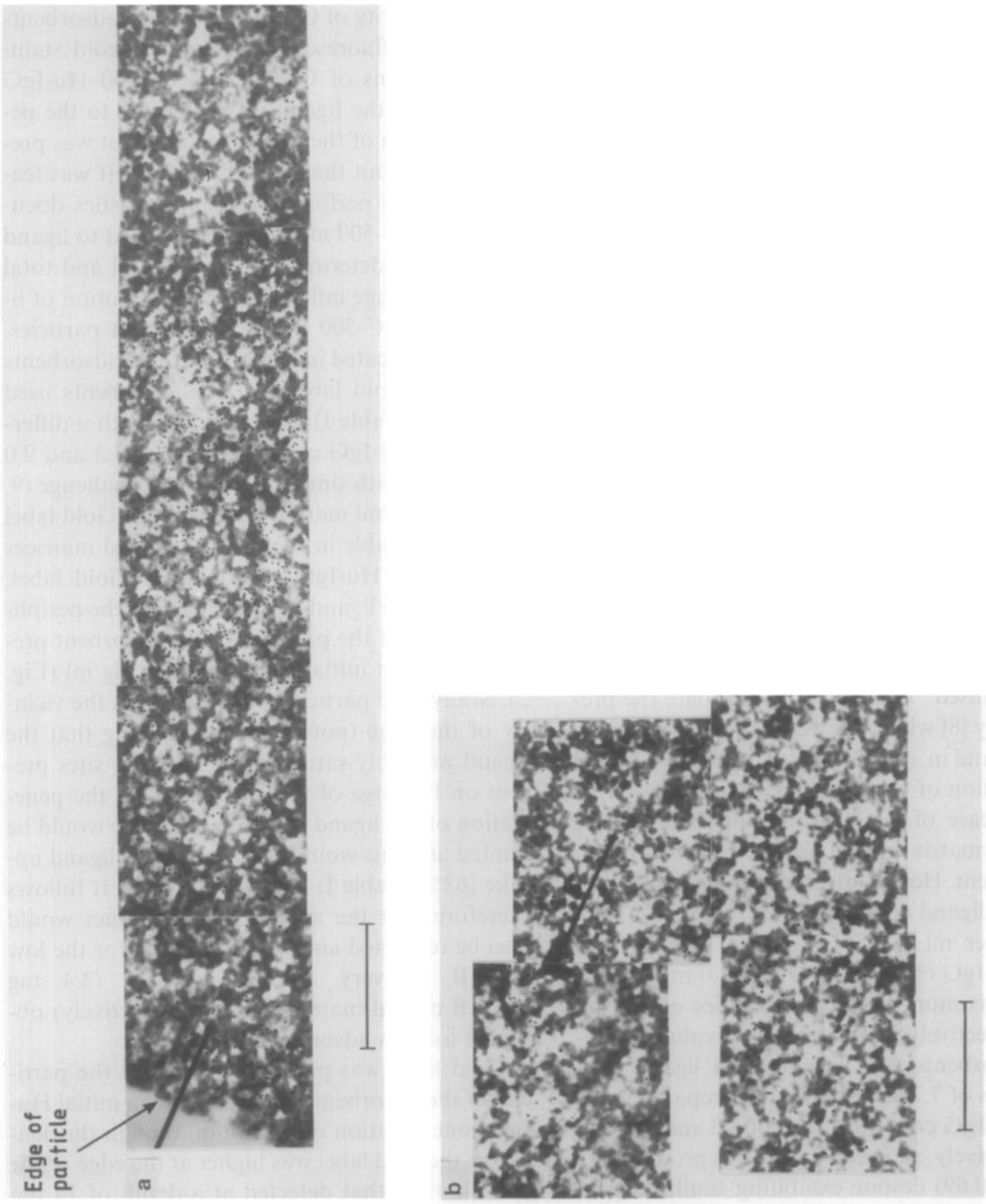

Fig. 3. Distribution of ligand within C-500 immunoadsorbent prepared with an initial challenge of 3.3 mg Hu-IgG per ml. (a) A composite photograph of sequential electron micrographs showing the distribution of gold label from the edge of the particle; (b) the distribution in the same particle at a depth of 14 μm. Photographs of the particle at low magnifications showing the two positions of the pointer were used to measure the distance between (a) and (b). Bar marker represents 500 nm.

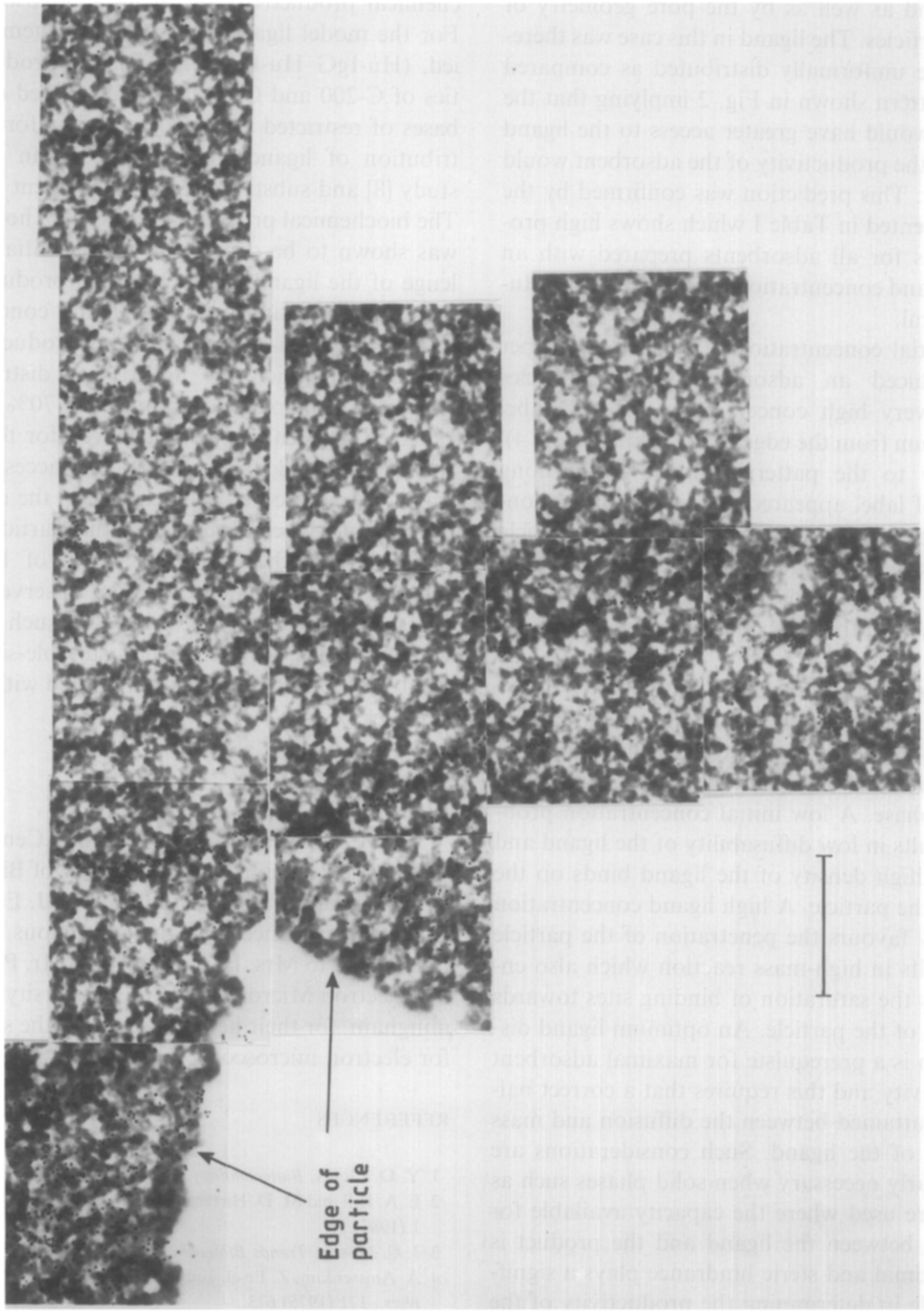

Fig. 4. Distribution of ligand within C-500 immunoadsorbent prepared with an initial challenge of 9 mg Hu-IgG per ml. The composite photograph shows the distribution of gold label from the edge of the particle as well as a scan of the streaks of label which appear to form a continuous barrier along the contour of the particle. Bar marker represents 500 nm.

within the particle (Fig. 3a and b, respectively). The density of gold label decreased further as the depth within the particle increased probably due to steric hindrance imposed by previously coupled ligand as well as by the pore geometry of C-500 particles. The ligand in this case was therefore more uniformally distributed as compared to the pattern shown in Fig. 2 implying that the product would have greater access to the ligand and that the productivity of the adsorbent would be higher. This prediction was confirmed by the data presented in Table I which shows high productivities for all adsorbents prepared with an initial ligand concentration of 2.5 and 3.3 mg Hu-IgG per ml.

An initial concentration of 9 mg Hu-IgG per ml produced an adsorbent whose particles showed very high concentration of gold label within 2 μm from the edge of the particle (Fig. 4). Contrary to the patterns shown above, long streaks of label appeared to form almost a continuous barrier along the contour of the particle beyond which very few gold particles could be detected. The restricted penetration of the ligand would explain the lower performance of this adsorbent.

It appears therefore that the dynamics of the coupling reaction between the ligand and the activated diol sites of the solid phase determine the final location of the ligand within the particles of a solid phase. A low initial concentration probably results in low diffusability of the ligand and hence a high density of the ligand binds on the edge of the particle. A high ligand concentration probably favours the penetration of the particle but results in high-mass reaction which also encourages the saturation of binding sites towards the edge of the particle. An optimum ligand distribution is a prerequiste for maximal adsorbent productivity and this requires that a correct balance be attained between the diffusion and mass reaction of the ligand. Such considerations are particularly necessary when solid phases such as C-500 are used where the capacity available for reaction between the ligand and the product is not maximal and steric hindrance plays a significant role in determining the productivity of the solid phase.

CONCLUSIONS

We have demonstrated that ligand location is an important parameter for determining the biochemical productivity of an immunoadsorbent. For the model ligand–biomolecule system studied, (Hu-IgG–Hu-IgG MCAB), the productivities of C-200 and C-1000 were explained on the bases of restricted penetration and uniform distribution of ligand, respectively, in an earlier study [8] and substantiated in the present paper. The biochemical productivity of C-500, however, was shown to be dependent on the initial challenge of the ligand used. Optimum productivity was achieved using an initial ligand concentration of 3 mg Hu-IgG per ml. This produced adsorbents in which the ligand was distributed throughout the particles, though the 70% of the expected physical capacity achieved for this adsorbent imposed some hindrance to access. This was demonstrated by the decrease in the density of the gold label observed as the particle was scanned from the edge to a depth of 14 μm. Though such dependence was not observed with C-200 or C-1000, it is feasible that such effects would become apparent when suitable-sized ligand–biomolecule systems were tested with these matrices.

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