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IMMUNOCHEMICAL CHARACTERISTICS AND PREPARATIVE APPLICATION OF AGAROSE-BASED IMMUNOSORBENTS[1,2]

J. W. EVELEIGH and D. E. LEVY

Biology Division
Oak Ridge National Laboratory
Oak Ridge, Tennessee 37830

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The physical and chemical properties of immunosorbents prepared from Sepharose 4B have been studied, with the objective of improving their utility in preparative applications. The most significant factors influencing efficient use of these immobilized reagents are loss of immunochemical reactivity with increasing protein substitution and the restrictions imposed by the porous nature of the support. Similar effects are observable with controlled-pore glass matrices, but they are of less significance when solid, nonporous supports are used. Considerable improvements in operational efficiencies have been obtained by limiting the amount of protein immobilization and restricting reaction to the more accessible surface regions of the porous supports. Improved operating protocols based on these principles have been adapted to automated chromatographic systems, resulting in a fourfold increase in productive capacity. With the addition of the continuous dialysis and concentration system described here, routine daily production of gram quantities of specific antibodies from antisera becomes possible. Similarly, with a knowledge of the basic characteristics of these immunosorbents, it has been possible to increase greatly the efficiency of small-scale separations and purifications of immunological reagents from limited sources. The general approach toward the practical exploitation of immunosorbents for preparative applications is discussed in the context of these findings.

INTRODUCTION

The general utility of immunosorbents for the specific separation of antibodies and antigens is widely accepted in current biochemical and immunological research. The dramatic increase in the development and use of solid-phase immunoassays testifies to the many inherent advantages offered by immobilized reagents in analytical applications. However, there is an increasing awareness of the potential of these immobilized reagents in preparative-scale separation and purification techniques that are an essential part of many programs. The high specificity and the relative simplicity of

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these separation methods, compared with established chromatographic processes, combine to ensure their continued development and application.

For the past seveal years this laboratory has been actively engaged in the exploitation of immunosorbent technology for the separation of antigens related to malignant diseases (1). Automatic recycling systems have been developed that enable the separation and purification of specific antibodies and antigens to be undertaken routinely (2,3). The effective utilization of these systems, and of the potential capabilities of immunologically applied biospecific adsorption in general, depends significantly on knowledge of the inherent characteristics of the insolubilized reagents. This study was undertaken in an effort to define these characteristics more rigorously and to exploit them more effectively in practice.

Our investigation has concentrated upon the properties and use of 4% agarose-derived immunosorbents, as it appears from the current literature and from our own experience that Sepharose 4B is the most favored and widely employed support material. This acceptance is due to the ready availability and relative inertness of the support and the simplicity of the derivatization procedure using cyanogen bromide (CNBr) (4). However, it is not an ideal support, as these results will show, and its inherent properties dictate certain restrictions in its application if the full potential of the technique is to be realized. To emphasize alternative approaches, this support is compared with other materials, including high-density dextrans (Sephadex G-25) and porous and solid glass for specialized applications.

Knowledge of the limitations imposed by the characteristics of support materials has stimulated investigations of further innovations to improve their performance. Although essentially consisting of changes in operating protocols, these have resulted in an increased efficiency of the preparative recycling systems and a marked improvement in the yield and quality of the products. The incorporation of ancillary devices to provide continuous dialysis and concentration has added a further desirable feature to the original preparative instruments. It is hoped that this experience will facilitate development and wider application in other areas of contemporary biochemical separation programs.

MATERIALS

Sepharose 4B and Sephadex G-25 were obtained from Pharmacia Fine Chemicals, Inc.; the Sepharose was used as received unless otherwise noted. Alkylamine Zir-Clad controlled-pore glass (CPG 1350) was manufactured by Corning Biological Products and obtained from Pierce Chemical Company. Solid glass spheres, an industrial product of the 3M Company

(Superbrite Type 150–5005), had a mean diameter of 70 μm and were refluxed in concentrated nitric acid before use.

Glutaraldehyde, as a 10% aqueous solution, was obtained from Electron Microscopy Sciences. 3-Aminopropyltriethoxysilane and divinyl sulfone were purchased from Aldrich Chemical Company. Dimethyl suberimidate dihydrochloride was a Pierce Chemical Company product, and CNBr was obtained from Eastman Kodak Company.

Human albumin (HA) was obtained from Behringwerke Ag as their "100% electrophoretic purity" grade. The antiserum used was obtained from a single goat immunized on a weekly schedule with 5 mg HA containing incomplete Freund's adjuvant. The different batch numbers referred to in the text relate to sequential weekly bleedings; the antibody titers therefore vary accordingly. Goat IgG was prepared from the crude antisera by chromatography on DEAE-cellulose using 0.02 M phosphate buffer (pH 8.0) for elution. Purified anti-HA antibody was obtained from pooled antisera by recycling affinity chromatography; it was estimated to have a purity better than 95% by radioimmunoassay.

^{125}I-labeled solutions of HA and goat IgG were prepared by the iodine monochloride method of Helmkamp et al. (5) and purified by Sephadex G-25 chromatography using phosphate buffer. The specific activity of these stock solutions ranged from 10 to 100 μCi/mg; working "spiked" solutions were prepared by dilution with nonradioactive material. ^{125}I activity was measured in a Nuclear Chicago gamma counter, and protein concentrations were estimated by optical absorbance at 280 nm or by the Lowry protein method.

The "phosphate buffer" referred to in the text is 0.1 M (pH 7.0) containing 0.1% sodium azide. Ammonium thiocyanate (NH_4SCN) solutions were prepared by dissolving the required amount of "Certified A.C.S." reagent (Fisher Scientific Company) in phosphate buffer. Tween 80, a nonionic detergent, was obtained from ICI Unites States, Inc.

METHODS AND RESULTS

Preparation of Immunosorbents and Effect of Increasing Bound-Protein Concentrations

During the past 5 years we have successfully prepared immunosorbents based on a wide variety of support matrices using both conventional and esoteric chemical methods. Immobilization of antibodies and model antigens, with retention of immunological reactivity, has been achieved on nylon, polycarbonate, cellulose derivatives including hollow-fiber devices,

and commercially available supports such as polyacrylamide, agarose, and various glasses. We have also investigated the preparative application of proteins immobilized by general and selective cross-linking. Except for the latter methods, which tend to be somewhat wasteful of immunological reagents, most are suitable for use as immunosorbents with, however, varying degrees of efficiency and operational facility.

Because of the relative ease of derivatization, overall suitability for preparative application, and general availability of consistent high-quality material, we have most thoroughly investigated the immunosorbents prepared from Sepharose 4B. However, since controlled-pore glass is more physically rigid, it offers several advantages over the gel formulation, while solid glass and, to a lesser extent, Sephadex G-25 have provided low-capacity nonporous supports.

Sepharose 4B and other agarose gels are conveniently activated by treatment with CNBr under alkaline conditions (4). The reaction is performed in practice by adding either solid CNBr or a solution in water or organic solvents to a cooled, stirred suspension of Sepharose. The alkaline conditions are maintained by either titrating with NaOH solutions (4) or performing the reaction in a strong alkaline buffer (6,7). The amount of CNBr used is far greater than that required for a stoichiometric reaction, owing to a rapid hydrolysis under these conditions.

Although the activated product can be stored, for maximal efficiency it is advisable to couple to the protein immediately after washing with cold water. This coupling is performed under mildly alkaline conditions in the cold for several hours. To avoid the somewhat hazardous activation procedure, stabilized activated Sepharose is available from the manufacturer; in preparing large batches for preparative applications, however, it is more economical to undertake the reaction (cost of raw materials being reduced by a factor of 10 for equivalent amounts). Alternative activation procedures using acetonitrile (7) or *N*-methylpyrrolidone (8) solutions of CNBr have not, in our work, significantly improved activation efficiency.

After they are washed to remove unreacted protein, the substituted gel beads are treated with a low molecular weight amine solution to inactivate any residual cyanate or imidocarbonate groups that could subsequently bind unwanted protein. We have used a solution of ethanolamine (pH 8.0) for this purpose rather than glycine or ammonium hydroxide to obviate the introduction of ionically charged groups. After it is washed further, the immunosorbent is ready for use or for storage in phosphate buffer in the cold for long periods of time.

The use of molecular extenders to increase the distance between the inert matrix and the ligand, although of reported importance in affinity chromatography of enzymes and receptor molecules, does not significantly

improve the performance of immunosorbents. It is doubtful if the extra effort involved in preparing these active derivatives (9) is justifiable for preparative applications due to the minor increases in reactive capacities.

Although it is possible to directly activate dextrose supports such as Sephadex by the CNBr treatment, the resulting coupling capacity is very low. It is to be expected that the particle size will have a marked effect on the amount of protein bound, but even using the superfine grade of G-50 (10–40 μm), only on the order of 70 μg IgG/ml was achieved. Prior treatment of the Sephadex with epichlorohydrin and phloroglucinol (10) with subsequent treatment with CNBr or divinyl sulfone, however, raised the amount linked to the order of 200 μg IgG/ml Sephadex G-50.

It is necessary to derivatize glass prior to activation and coupling of proteins to the support. This is conveniently accomplished by incubating the acid-washed glass with an acetone solution of 3-aminopropyltriethoxysilane for 24 h at room temperature (11). In the case of controlled-pore glass, however, it is more convenient to use the commercially available alkylamine-derivatized material for routine application. After it is washed to remove excess silane, the aminated glass is reacted with either cold 1% aqueous glutaraldehyde or dimethyl suberimidate as a 0.2 M solution in methanol–borate buffer (pH 9) for several hours. The activated glass is washed and then incubated overnight under mildly alkaline conditions with the protein to be coupled.

The problem of inactivating excess unreacted sites is more difficult with glass than with Sepharose. This is particularly apparent when glutaraldehyde is used, as the Schiff's base formed in the reaction with amines is known to be somewhat dissociable. Although proteins are effectively immobilized, probably due to attachment by multiple linkages, monovalent blocking reagents are notoriously labile, regenerating active groups to subsequently bind undesired proteins. To overcome this lability, reduction of the Schiff's base formed with an amine with sodium borohydride can be employed. However, we have found after experimentation with a variety of compounds, that reaction with an excess of an aqueous solution of hydroxylamine (pH 8) to form the oxime with remaining aldehyde groups is equally effective. Dissociation of the Schiff's base formed with the silylamine by this nonreductive treatment is, of course, not prevented; the effect of the possibly regenerated amino group is, however, ionic and can be suppressed by high salt concentrations to inhibit subsequent nonspecific protein interactions. The imido ester linkage is nondissociable, and reaction with a simple amine is completely effective.

Our experience using glass has been that activation with glutaraldehyde is more effective than with suberimidate (and other undiscussed chemistries) from the aspect of capacity or amount of bound protein per unit volume of

support. From an immunochemical aspect, however, the potential of non-specific interactions is increased. Up to 20 mg IgG/ml controlled-pore glass can be immobilized, and on the order of 100 μg/ml on 70-μm solid glass. The amount of protein linked to the latter can be approximately doubled by prior etching of the glass in 2 N NaOH at 84°C for 1–2 h. These amounts are within the limits calculated for a monolayer of immunoglobulin distributed on the surface of solid spheres of this diameter.

Although published methods were used to prepare large batches (50–200 ml) of immunosorbent for later studies on column operating and equilibrium conditions, several questions are raised by employing these procedures. The foremost of these relates to the degree of protein substitution and its effect on immunochemical reactivity. In many applications a high degree of substitution is desirable, allowing the use of more compact column beds and permitting greater sample capacities. Moreover, efficient utilization of valuable antigens and antisera, immunized by this procedure, dictates minimal loss of reactivity and specificity.

To investigate this effect of degree of substitution, the activation and coupling procedures were adapted to small-scale operation. Packed Sepharose (1 ml) was dispensed as a 50% slurry into scintillation counting vials (~15-ml capacity). For subsequent activation with less than 100 mg total CNBr, the suspensions were prepared in 2.5 M potassium phosphate buffer (1.65 mol K_3PO_4 + 0.85 mol K_2HPO_4 per liter); otherwise aqueous suspensions were dispensed. The stirred suspensions were cooled in an ice-water bath and reacted for 10 min with an aqueous solution of CNBr or titrated with 1 N NaOH to maintain pH 11.0–11.5 when solid CNBr was used. After activation the Sepharose was transferred to a small sintered-glass funnel by washing rapidly with cold water and finally with 0.25 M $NaHCO_3$ solution.

Coupling was performed by adding the moist cake directly to ^{125}I-spiked protein in 0.25 M $NaHCO_3$. The reactants were stored in the cold room, with occasional shaking, for a minimum of 12 h. After the beads were washed, treated with 1 M ethanolamine (pH 8.0) for 30 min, and further washed by centrifugation, the incorporation and/or reactivity was determined by counting the gels directly or after reaction with labeled antigen.

To determine the effect of the initial CNBr concentration on the amount of protein subsequently covalently linked to the support, spiked goat IgG (batch 3) was used. Solutions were prepared to contain a total amount of protein that was in excess of that predicted for complete reaction, e.g., 20–50 mg in 4 ml of coupling buffer. The results are shown in Fig. 1. The concentration of CNBr is the initial concentration in the reaction mixture; below 50 mg/ml the reagent was added in aqueous solution, above as a solid aliquot.

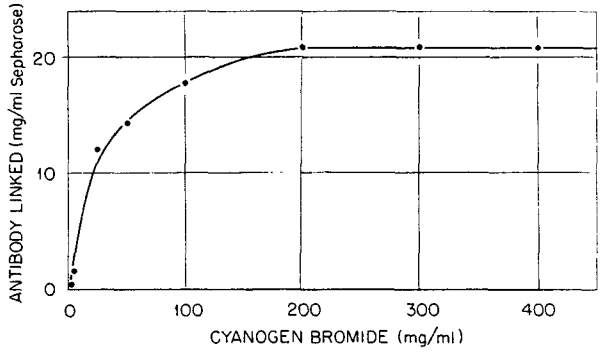

FIG. 1. Covalent linking of antibody to Sepharose 4B as a function of CNBr concentration.

The results clearly show a maximal reaction of 21 mg IgG/ml packed Sepharose with an initial concentration of approximately 200 mg/ml CNBr. Increasing the amount of reagent over this does not yield further coupling, but reduction results in effectively lowering the IgG incorporation.

The stability of an aqueous CNBr solution was also tested during this experiment, both as a check on the experimental protocol and as a potential manipulative saving for future work. A 100-mg/ml solution was stored at room temperature (crystallization occurs in the cold) and used for activation after periods of 24 and 48 h. A 1-day storage reduced its reactivity to 95%, compared with that of the freshly prepared solution, and a 2-day storage reduced it to 61%. Therefore, it seems impractical to prepare aqueous stock solutions of this reagent if reproducible immunosorbents are required. The long-term stability of organic solvent solutions, however, has been reported to be satisfactory (7) for convenient soluble storage of the reagent.

To compare the effect of the amount of protein covalently bound per unit volume of support on its immunochemical reactivity, there are two alternative approaches to preparing the immunosorbents:

1. Limit the activation by restricting the total amount of cyanogen bromide and couple with an excess of protein.
2. Activate with a constant high concentration of cyanogen bromide and limit the amount of protein available for coupling.

The experimental protocol was therefore designed to include these variables: Two sets of 1-ml aliquots were activated and coupled with lightly spiked goat anti-HA IgG (batch 4). In the first set (tubes 1–6, Table 1) the total amount of CNBr was increased from 10 to 200 mg and the 1gG was provided in excess of that required. The second set (tubes 7–11, Table 1) was

TABLE 1. **Effect of Increasing Substitution of Antibody on Its Immunochemical Activity**[a]

Tube no.	Total CNBr (mg)	Total IgG (mg)	Coupled IgG (mg)	Bound HA (mg)	Specific activity (mol HA/ mol IgG)
1	10	30	1.496	0.328	0.54
2	25	30	8.382	1.502	0.44
3	50	30	14.84	1.869	0.31
4	75	30	16.52	1.876	0.28
5	100	30	21.43	1.860	0.21
6	200	30	22.43	1.915	0.21
7	100	1	0.950	0.162	0.42
8	100	2.5	2.347	0.359	0.38
9	100	5	4.553	0.734	0.40
10	100	10	8.709	1.263	0.36
11	100	20	14.67	1.808	0.30
12	100	0	0	0.011	—

[a] One-milliliter aliquots of packed Sepharose activated and coupled with anti-Ha IgG under various conditions and their subsequent reactivity with HA.

activated with 100 mg CNBr and coupled with increasing amounts of antibody. A control was included (tube 12, Table 1) in which activation was performed but no protein was coupled. After coupling overnight in the cold, the immunosorbents were washed and treated with ethanolamine before the amount of coupled IgG was determined. They were then reacted with excess spiked HA overnight; after they were washed the amount of bound HA was determined. The results are shown in Table 1.

It is evident from these results that the amount of covalently bound antibody per unit volume of support, and to a lesser extent the procedure used, influences the subsequent reactivity of the support to antigen. However, it must be stressed that these results are to be considered only relatively, since the efficiency of coupling and resultant reactivities are obtained solely under the conditions reported with this particular antiserum–antigen system. This is particularly important in interpreting the specific immunological activities of the samples; the values, although highly significant from the relative viewpoint, are absolute only in that they apply to the particular preparation of goat IgG used. In later experiments with purified antibodies the maximal specific activity found with lightly loaded immunosorbents was 1.25 (expressed as a molar ratio). If antibodies are assumed to be divalent, this is equivalent to a maximum efficienty of ~60%; efficiency falls to around 25% when the amount of antibody is increased to 10 mg/ml or greater.

A similar effect of loss of immunochemical reactivity with increased protein substitution was found with the other supports. On porous glass, antibody showed higher efficiencies than similar concentrations on Sepharose 4B, reaching specific activities of 1.50 when less than 1 mg of IgG was linked per milliliter of glass. In the case of solid glass, provided an expected monolayer was not exceeded, the dependence of activity on antibody density was not nearly so pronounced. Specific activities between 1.00 and 1.35 were found at all levels, and it appears that other factors, or variables in the activation procedure, have a greater effect on reactivity. Immunosorbents prepared with Sephadex have repeatedly shown a low efficiency of immunological reactivity; despite potential physical advantages of the support, their use has been consequently restricted to analytical and chemical kinetic applications.

These effects have been confirmed using other antisera and antigens, including a multicomponent serum protein system. Immobilized proteinaceous antigens exhibit varying degrees of density dependence, although in these multivalent conditions the effect is less predictable. The implications with regard to efficient use of reagents and possible explanation of the effect are considered in the Discussion section.

Immunochemical Characterization of Immunosorbents

To utilize fully the potential of an immunosorbent for the production of reagents by affinity chromatography, it is preferable that its characteristics be determined prior to application. An empirical approach with a small column of the immunosorbent can yield valuable guidelines for choosing capacity and operating conditions for larger preparative systems, but optimization will require more basic studies. The time spent is inevitably saved by the increased yield or purity resulting from the simplest of these investigations. Many of the basic properties such as specificity, reaction rate, and affinity constant depend on the individual antibody–antigen system being explored and indeed on the particular antiserum–antigen preparation employed. However, other characteristics are more general in that they depend on the choice of support material and the conditions under which it is used.

Thus, although only a single immunological system is reported here, and the absolute characteristics are relevant to this system only; the more general properties are applicable to other systems using porous and solid supports.

Affinity Constant: Determination and Practical Effects. The affinity constant can be conveniently determined with immunosorbents by measuring the bound-to-free ratio of the soluble species and presenting the data in

an acceptable form. Radioactively labeled reagents, which simplify the procedure, were used in these determinations. The general procedure was as follows: Equal aliquots (5 ml packed volume) of bulk-prepared recycled immunosorbent of goat anti-HA IgG were dispensed into 40-ml plastic centrifuge tubes. To these were added known amounts of spiked HA, and the volume was adjusted to 25 ml with phosphate buffer. The tubes were capped and agitated gently on a rocking table for at least 2h at room temperature and even longer in low-temperature determinations. Samples of the supernatant were taken after low-speed centrifugation and counted for remaining antigen concentration. Variations of this method, including reaction in counting tubes and direct counting of immunosorbent, were also employed in the case of solid supports to improve precision. To determine the effect of chaotropic ions (12), the reacted immunosorbents were equilibrated with appropriate dilutions under similar conditions.

In expressing the results in a suitable form, the concentration of insolubilized antibody is not a workable parameter; it is therefore expressed on a molar basis as moles per liter of Sepharose or in mass units as micrograms per milliliter of Sepharose. However, albumin concentrations are calculated conventionally. The most useful way to present results, obviating any bias of the higher concentrations, is via the Scatchard plot (13). In this, $\bar{v}$, the molar ratio of bound antigen to linked antibody, is plotted against $\bar{v}$/[antigen]. A typical result is shown in Fig. 2 for equilibrium at pH 8.0 in 0.1 M phosphate, 1.5 M NH_4SCN, and 2.5 M NH_4SCN at 25°C. The immunosorbent used in this experiment was from an early bleeding of an immunized goat (batch 2); it contained 5.3 mg IgG/ml packed Sepharose (3.2×10^{-5} mol/liter). In Scatchard plots, the intercept of the curve on the abscissa gives n, the number of sites, which for a pure, fully active, divalent antibody theoretically should equal 2.0. The intercept on the ordinate is equal to Kn, from which K can be estimated. Thus, in this particular determination, if $n \simeq 0.35$, K in phosphate (pH 8.0) $\simeq 3.3 \times 10^7$.

Heterogeneity of the antibody is indicated by nonlinearity in the Scatchard plot, as is evident in this sample of antibody in the chaotropic ion solutions. Preparations of antibodies from later bleedings showed greater heterogeneity, even in phosphate buffer; however, their association constants were generally higher than in this example.

A more practical form of presentation of the data is the "direct plot," in which $\bar{v}$ is given as a function of [antigen]. These parameters can of course be expressed in more practical mass–volume units, allowing for direct experimental interpretation. Figure 3 is a direct plot of a composite of determinations demonstrating the effect of eluent solutions at pH 7.0 and 25°C, in free-antigen concentration units of micrograms per milliliter and bound

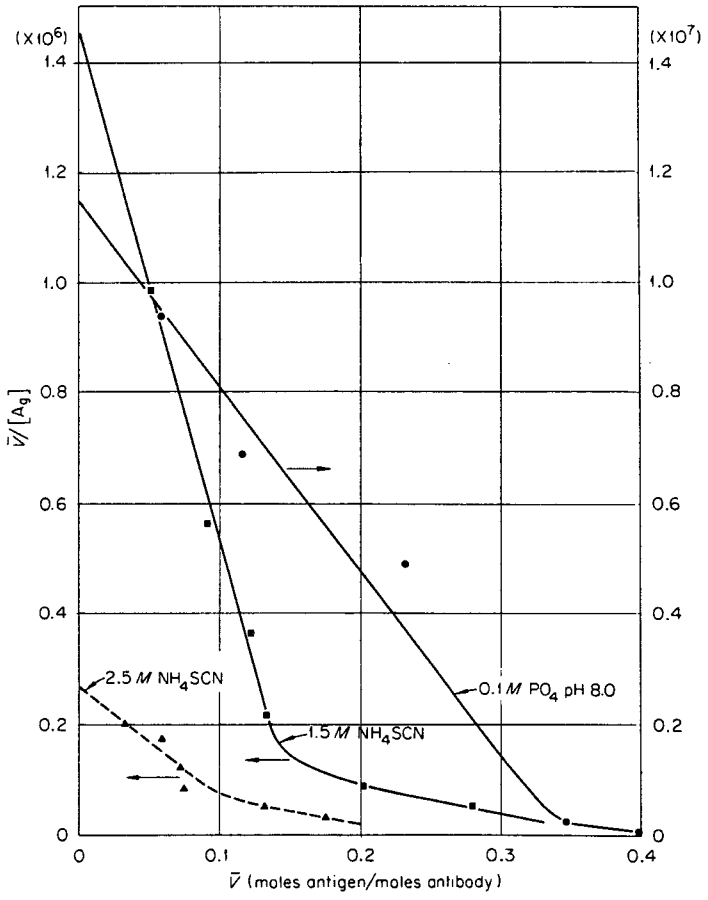

FIG. 2. Scatchard plot of anti-HA Sepharose reaction with HA at pH 8.00 and 25°C.

units of micrograms per milliliter of packed immunosorbent; it was constructed from values of association constants determined as previously described.

From graphs such as this, the relative "efficiency" of eluents can be directly determined in practical terms. For example, from Fig. 3, it becomes obvious that 1.5 M NH_4SCN will release more bound antigen at any given free-antigen concentration than will 6 M urea. Moreover, the quantity released or adsorbed can be directly extrapolated in convenient units. The graphs will thus allow prediction of these effects that can be employed for

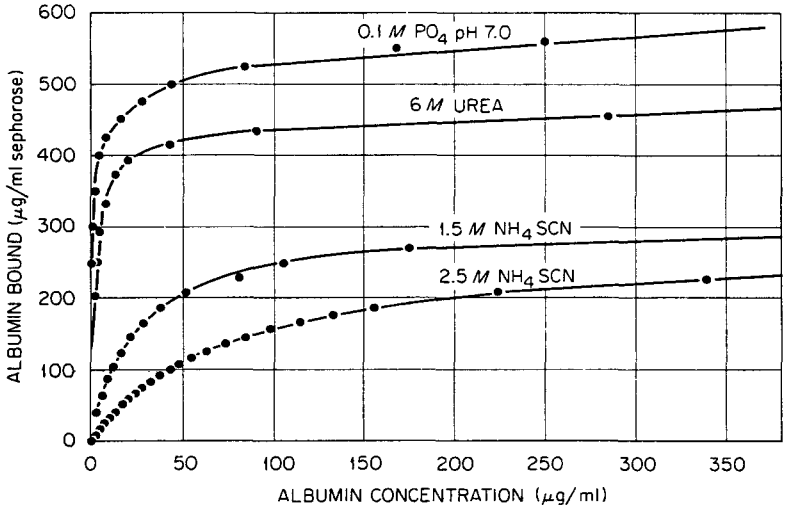

FIG. 3. "Direct plot" of HA bound to specific immunosorbent expressed in weight per volume units as derived from affinity constants.

subsequent optimization in utilizing the immunosorbent for practical purposes, as well as for obtaining a more thorough insight into the principles involved.

As an example of this approach, and using Fig. 3 as a basis, we can predict the adsorption capacity of this particular immunosorbent at various concentrations of antigen. From the graph we find that the maximum capacity, at very high concentrations of albumin, is ~625 μg/ml immunosorbent in phosphate buffer. If we were now to prepare solutions of albumin containing a total amount 10 times in excess of this, e.g., 6.25 mg, in various volumes of phosphate buffer and allow these solutions to come to equilibrium with 1 ml of immunosorbent, then the amount of albumin bound would be as shown in Table 2.

Similarly, if one were to treat this "saturated" immunosorbent, containing 625 μg albumin/ml, with various volumes of 2.5 M NH_4SCN, it can be predicted that the amounts of albumin released into solution would be as listed in Table 3.

It is very evident from these tables that the efficiency of adsorption of an antigen from solution by an antibody immunosorbent is highly dependent on its concentration. Conversely, the volume of eluent will directly affect the yield on subsequent desorption. The implications arising from this in practical applications are considered in the Discussion section.

TABLE 2. **Prediction of Amount of Albumin Bound to 1 ml Immunosorbent at Various Concentrations of Albumin in 0.1 M Phosphate (pH 7.0) at Equilibrium at 25°C**[a]

Volume of phosphate buffer[b] (ml)	HA concentration (μg/ml)	Bound HA (μg)	Maximum uptake (%)
20	312	570	91.2
50	125	535	85.6
100	62.5	515	82.4
200	31.2	482	77.1
500	12.5	438	70.1
1000	6.2	410	65.5
2000	3.1	382	61.1
5000	1.2	280	44.8

[a] From Fig. 3.
[b] Containing 6.25 mg total HA.

TABLE 3. **Prediction of Amount of Albumin Released from 1 ml Saturated (625 μg/ml) Immunosorbent by Various Volumes of 2.5 M NH_4SCN (pH 7.0) at Equilibrium at 25°C**[a]

Volume of 2.5 M NH_4SCN (ml)	Unbound albumin (μg)	Percent released
1.17	400	64.0
2.12	425	68.0
3.38	450	72.0
5.27	475	76.0
8.06	500	80.0
12.50	525	84.0
19.64	550	88.0
33.80	575	92.0
75.00	600	96.0
122	610	97.6
310	620	99.2

[a] From Fig. 3.

Finally, the effects of temperature and pH on the affinity constants, and the resulting implications for practical exploitation, must be considered. A large amount of effort was directed toward studying these variables, but for practical improvement the results were disappointing—at least with the HA system. As expected, the association constant increases at lower reaction temperatures and decreases at elevated (37°C) incubation conditions. However, the magnitude of the difference is slight, being only a factor of 2 or so for the extremes. This minor improvement in affinity is practically negated, however, by the long period (more than 24 h) necessary to achieve equilibrium. Temperature cycling for preparative purposes is impractical in this system, and it has become our standard practice to operate all production applications at room temperature.

The effect of pH is more pronounced, and indeed extremes of pH have been widely used in affinity chromatography. Association constants reach a maximum between pH 7 and 8 and fall drastically below pH 4 and above pH 10. In many antibody–antigen systems, however, these acidic or alkaline conditions are deleterious to the antigen tertiary structure and can also irreversibly affect the antibody. Even with relatively stable proteins, such as HA, some deterioration of immunosorbent capacity is noticeable on continued recycling with glycine hydrochloride buffer (pH 3.0). For this reason, we have tended to use exclusively chaotropic ion eluents in which the pH is maintained between 6 and 8.

The effect of pH is slight on both adsorption and desorption within this narrow range. A small advantage can be gained by adsorption at pH 8.0 rather than at lower values, as it appears that more of the lower affinity antibody species become reactive under these conditions. Conversely, slightly higher yields of desorbed antigen are obtained by eluents at the lower end of the range.

Rate of Reaction and Diffusional Effects. The overall reaction rate of an immunosorbent with a soluble reactant is a function of both chemical and physical kinetic factors. Immunochemically, the reaction follows second-order kinetics, which means that the velocity is concentration dependent. The effective concentration of the immobilized species is, as described earlier, influenced by the degree of support activation, the amount of coupled protein, and its specific activity. For a given immunosorbent, however, this concentration can be assumed to be constant in considering the practical effects of the reaction kinetics. Thus the immunochemical rate becomes proportional to the soluble component's concentration, assuming comparable environmental conditions.

A further simplification becomes possible when either component is in large excess relative to the other. Under these conditions the extent of reaction can be pragmatically considered to be exponential with time, i.e., following pseudo-first-order kinetics. This approach becomes useful in

comparative studies, although it is directly applicable only in the depletion of trace soluble material from a sample. The efficient utilization of reagents normally dictates that the relative amounts of reagents approach unity in preparative applications.

The immunochemical reaction is, however, rarely the limiting parameter, for physical constraints involving access of the soluble components generally determine overall adsorption rates. To provide a large surface area in a minimal volume of immunosorbent it becomes necessary to use porous supports in most preparative applications. The internal geometry of the pores can be considered highly tortuous and certainly heterogeneous. This type of structure enhances steric hindrance and, because mixing can be considered negligible within the support, dictates diffusion-dependent kinetics. These diffusional constraints involve not only mass transfer considerations but, in the case of high molecular weight proteins, rotation or orientation restrictions.

To demonstrate the effect of diffusion, periodic sampling and assay of excess free antigen during an equilibration experiment is adequate. Figure 4 is an example of a typical result obtained with Sepharose containing 5 mg antibody/ml reacted with an excess of labeled HA, first in phosphate buffer and subsequently in 2.5 M NH_4SCN. It is relevant to note that the adsorption rate is practically identical to the desorption rate, indicating that antigen diffusion is involved in the process.

The pseudo-first-order rate constant for the earlier portion of the reaction is 2.4×10^{-3} sec^{-1}, corresponding to a half-reaction time of 4.7 min. However, the last 15% of the reaction is much slower, with a constant of 6.3×10^{-4} sec^{-1} ($t_{1/2} = 18.4$ min). The numerical values quoted bear little relationship to the absolute rate constant; they are valid only for the concentrations used under these particular experimental conditions. Other experiments, which will be reported elsewhere, indicate absolute rate constants on the order of 10^6 liters mol^{-1} min^{-1}. The earlier, more rapid reaction is approximately the same value as that found using Sephadex under similar conditions, thus indicating surface reaction. The slower rate can therefore be interpreted to represent the diffusion of antigen within the pores of the support. It appears from this and similar data that about 80% of the reactive antibody population can be considered readily accessible when Sepharose is used as the support. Controlled-pore glass with a nominal pore diameter of 1350 Å was only marginally more effective than Sepharose, but this may be related to the greater heterogeneity in particle size observable in this material. Solid glass gave the fastest overall rates, which is, of course, to be expected in a nonporous support.

These results indicate a potential means whereby the diffusional effects can be reduced, thus offering improved time efficiency for preparative applications. If reaction could be restricted to the outer layer of the porous

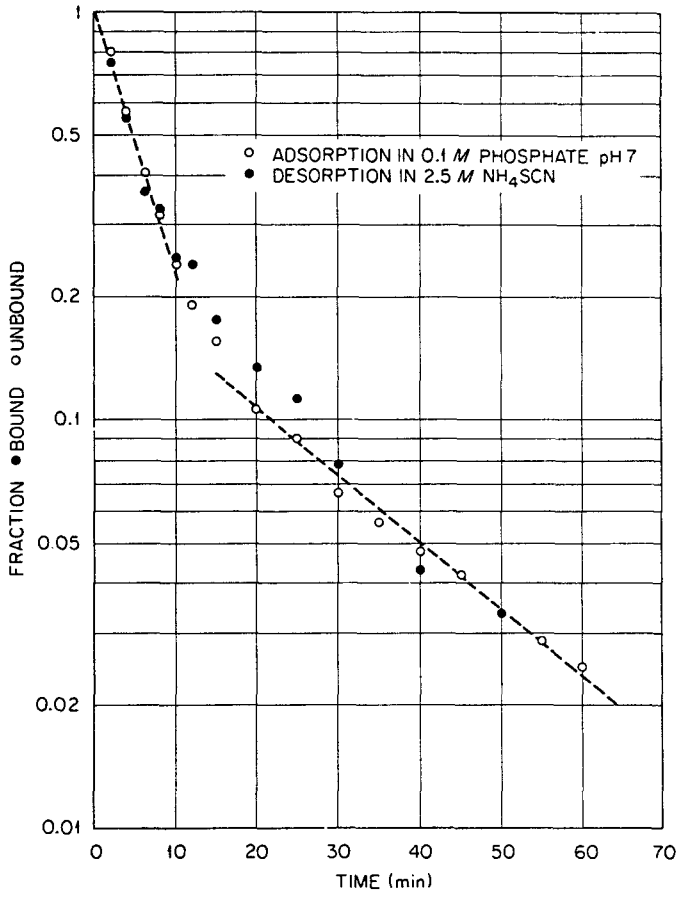

FIG. 4. Adsorption and desorption of HA from its specific immunosorbent under stirred-batch conditions.

support, either by curtailing the total amount of soluble component available or by reducing the time allowed for reaction, the adverse diffusional effect could be avoided.

To test this concept, the effect of sample load on the kinetics of reaction was detemined using whole serum containg labeled antibodies and a Sepharose antigen immunosorbent. To simulate practical use of separation, whole serum was used instead of purified antibody. Fluid from a stirred 50-ml reaction vessel was pumped through a Teflon coil placed in the crystal well of the gamma counter and then returned to the vessel. Various amounts of goat antisera (batch 5) containing ^{125}I-labeled anti-HA IgG (batch 4) were made

up to 10 ml with phosphate buffer containing 0.5% Tween 80 (see following section) and circulated until the counting rate stabilized. Ten milliliters of a 50% slurry of HA–Sepharose was added, and sequential (fast print setting) counting commenced. A porous disk in the reaction vessel prevented the immunosorbent from leaving the chamber, thereby allowing the uptake of antibody to be followed dynamically.

The results are shown in Fig. 5, which is a composite plot of the unreacted fraction of soluble antibody against time, for increasing sample volumes. The "blank" plot, in which plain Sepharose 4B was used in place of immunosorbent, is included to indicate the mixing characteristics of the system; the ordinate in this plot is, of course, more correctly represented by the *unmixed* fraction rather than the unreacted fraction. It is apparent from these results that as the sample load is increased, the reaction rate is effectively decreased, particularly for the last 10% of the reaction. From an initial half-reaction time of 1.5 min (below 0.1 reaction fraction) with a 0.5 ml serum load, the $t_{1/2}$ increases to 7.6 min when volumes of 5 ml or greater are used.

This finding can be employed in designing operating conditions for maximizing production where time is of prime importance, and is explored more fully later. From a quantitative aspect, however, there is a loss in overall capacity of the immunosorbent by restriction of the sample to below

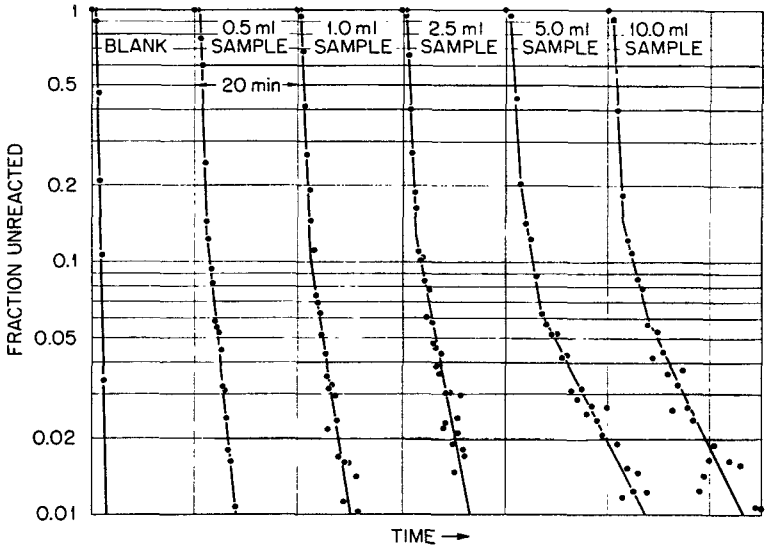

FIG. 5. Effect of increasing sample size on reaction of antibody with its specific antigen immunosorbent.

maximal. The potential of avoiding this small but significant wastage of reagent is provided by restricting the initial amount of protein used in preparing the immunosorbent. By this means the highest proportion of active species will be coupled to the outer, more accessible regions of the bead.

Nonspecific Adsorption. Ideally the support matrix of an immunosorbent should be completely inert and show no interaction with components of the sample being processed. This criterion is rarely achieved in practice, as derivatization of the support invariably introduces ionically reactive groups. In the case of CNBr-activated Sepharose these include positively charged urethane residues (14), or isourea groups when amine spacers are used (15). Aminated glass inherently contains residual amine groups and other reactive species dependent on the chemistry used for linking the protein. To add to the complexity of these potential ionic and chemically induced interactions, the hydrophobic properties of some supports and of the immobilized proteins can provide a further mechanism whereby nonimmunological reactions can occur on an immunosorbent.

These nonspecific interactions can be considered to consist of two major types: A nonreversible primary reaction that occurs in the first exposure of an immunosorbent to a complex biological sample, and a reversible, nonspecific adsorption of certain components that results in contamination of the eluted fraction.

The nonreversible adsorption of unwanted proteins is often due to inadequate blocking of reactive sites with a low molecular weight reagent. As previously mentioned, the instability of Schiff's bases formed in the reaction of glutaraldehyde with amines exemplifies this particular problem. The choice of an effective blocking reagent and sufficient exposure to ensure complete reaction does much to reduce this problem. However, there remains an as yet unexplained irreversible binding of specific protein to an immunosorbent on its first exposure to sample. This has been observed using many supports and occurs with both antigen and antibody immunosorbents. Because this "adsorbed" protein is not displaced by eluting agents, the practical effect is an apparent loss of preparative capacity as determined by the results of the first cycle using the immunosorbent. The amount of loss can be as much as 25% with highly loaded porous supports, but with solid glass it rarely exceeds 10%.

Reversible, nonspecific (nonimmunological) adsorption imposes the most significant problem in obtaining high-quality products using the method. Largely due to ionic and hydrophobic effects, the practical recourse is to suppress these contributions by suitable choice of nonionic blocking reagents and the use of buffers containing large amounts of salt and detergent. The positively charged urethane groups of CNBr-activated

Sepharose reportedly have been suppressed by treatment with blue dextran (14). We have found the ion exchange potential of residual alkylamine groups on glass to be significantly reduced by the modification of the phosphate buffer to 1 M with respect to NaCl. Although this higher salt concentration has a slighly chaotropic effect, the small loss in capacity is amply compensated by an increase in purity of the eluted species.

One of the less predictable effects is due to the fact that the immobilized protein itself can act as an ion exchange medium. In the preparation of specific antibodies to an acidic glycoprotein, for example, the preparation is invariably contaminated with nonspecific basic immunoglobulins. Conversely, antigens recovered from antibody immunosorbents are often contaminated with acidic components, notable albumin. We have found the incorporation of Tween 80 in the phosphate buffer used for washing and adsorption to be remarkably effective in reducing this undesirable adsorption.

The effect of adding this detergent to the samples and buffers used for specific antibody production with insolubilized antigen columns is shown in Table 4. In this experiment 5-ml columns of HA–Sepharose were employed to prepare specific antibodies from goat IgG (batch 3) using phosphate buffer and phosphate buffer plus 0.5%. Tween 80. The adsorbed antibodies were subsequently eluted with 2.5 M NH_4SCN, then coupled to activated Sepharose before their specific activities to albumin were determined. The results show a higher specific activity for the antibodies produced with the detergent-containing buffers than for those obtained with phosphate buffer alone. The most plausible explanation is that of contamination of the detergent-free buffer with inactive species of IgG, causing a lowering of the resultant specific activity.

TABLE 4. **Specific Activities of Anti-HA Immunoglobulins Produced from 5-ml Columns of HA–Sepharose Using Phosphate Buffer and Phosphate Buffer Containing 0.5% Tween 80**

0.1 M PO_4 (pH 7.0)		0.1 M PO_4 (pH 7.0) + 0.5% Tween 80	
Sample no.	$\bar{v}$ (mol HA/ mol IgG)	Sample no.	$\bar{v}$ (mol HA/ mol IgG)
1	0.986	5	1.238
2	0.952	6	1.231
3	1.164	7	1.238
4	1.091	8	1.290
	Mean 1.048		Mean 1.249

Column Operation

Although simple batch processing can be used in applying immunosorbents for preparative purposes, the column chromatographic method offers many advantages. Not only is it less demanding technically and more readily automated, it is inherently more efficient and adaptable to a variety of related separations. Efficiency is defined in terms of the most valuable resource and the objective of the process. Thus, in preparative applications time or manual effort is usually at a premium, and efficiency is related to yield or maximal output per unit time. However, it the supply of antibody is severely restricted, maximum utilization of this component becomes the overriding consideration. The optimization of any procedure will therefore depend on these factors in addition to the basic characteristics of the system and its reagents. In practice this implies that compromises will have to be accepted in formulating operational protocols to achieve the desired efficiency within a given application.

Conventional Chromatographic Operation. Adsorption chromatography is a relatively simple procedure in which the active species are specifically adsorbed by the immobile reagent and subsequently desorbed from the column by an eluent. Between adsorption and desorption the column is flushed with washing buffer to remove unbound material and eluent salts. For a given immunosorbent, the variables of this system are few and relatively simple compared with other chromatographic separations. They consist essentially of column dimensions; volumetric considerations regarding amounts of sample, eluent, and buffer washes to be used; and environmental conditions such as pH and temperature. The choice of these latter parameters is fortunately simplified in most applications by the previously mentioned findings that the effects of temperature and pH, within a restricted range, are only minor with regard to improving efficiency. Similarly, column geometry (ratio of length to cross-sectional area) becomes significant only as it affects physical constraints related to flow resistance rather than directly affecting efficiency.

The operational variables are thereby reduced to an empirical determination of the following:

1. Effective capacity of the column (i.e. sample load).
2. Volume of buffer wash.
3. Volume of eluent.
4. Volume of second buffer wash.
5. Effect of continued cycling.

It is conventional practice to operate the column at a constant flow rate so that the sample, buffer washes, and eluent volumes become directly

related to application time intervals. This flow rate is obviously a function of the column dimensions but is limited in practice by column resistance and the tendency of the softer porous gels to compact under hydrodynamic stress. The use of more rigid supports offers considerable advantages in this respect and is strongly recommended for high flow rates. It is to be noted that the degree of protein substitution of agarose gels influences these limitations, highly substituted supports possessing flow characteristics far superior to those of lightly loaded gels. It has been our experience, using 2.5-cm-diameter columns containing up to 200 ml of Sepharose immunosorbent, that flow rates between 0.5 and 5 ml/min provide reliable continuous operation without problems associated with compaction.

There are two alternative approaches for determining effective capacity of the immunosorbent column: either continuous application of the sample solution and assay for breakthrough of the active species, or sequential addition of increasing volumes with subsequent elution between additions. If the means are available for specific analysis of the adsorbed component, such as radioactively labeled material, frontal analysis provides the required adsorption efficiency data in a single experiment. The amount of adsorbed component relative to the volume of sample applied is obtained by difference, thus allowing a direct choice of sample load with the desired degree of efficiency. The alternative method of incremental sample additions not only is time-consuming but also requires that the effects of elution between aliquots be taken into consideration; it is therefore a more empirical procedure.

The volume of buffer wash following sample addition is largely determined by the degree of purity required in the separation. With porous supports, diffusion of inert species within the support can be expected to occur, giving rise to a gel filtration effect. The volume of buffer required to lower contamination to acceptable levels becomes a function of flow rate or exposure time under these conditions. The flushing of the column can be monitored with a UV flow cell for most practical applications, return to baseline values being indicative of washing efficiency. It must be realized from consideration of the affinity constant that some dissociation of the specifically adsorbed material is inevitable during the exposure to buffer. The amount lost is highly dependent on the affinity constant and heterogeneity of the antibody preparation, but it rarely exceeds 1 or 2% in the majority of applications using two or three column volumes of wash buffer.

The frontal analysis method is again preferable in determining a practical volume of eluent. Owing to the optical absorption of NH_4SCN in the UV, monitoring for protein directly is possible only with acid elution. This necessitates the use of radioactive labels or prior dialysis of the

UV-absorbing eluates before analysis. Continuous dialysis, utilizing hollow-fiber devices, has become a standard feature of our systems and can be used for these desorption studies. Figure 6 is an example of the desorption of albumin from a recycled 5-ml Sepharose anti-HA immunosorbent at a flow rate of 0.6 ml/min of three concentrations of NH_4SCN. The fraction of antigen remaining on the column is represented with respect to time and hence volume of chaotropic eluent. It can be seen that to recover 90% of the antigen requires nearly four column volumes of 2.5 M NH_4SCN, a finding to be expected from the basic characteristics discussed earlier. If only one column volume is used, the recovery falls to approximately 60% of the maximum. The unrecovered antigen is readsorbed in the succeeding buffer

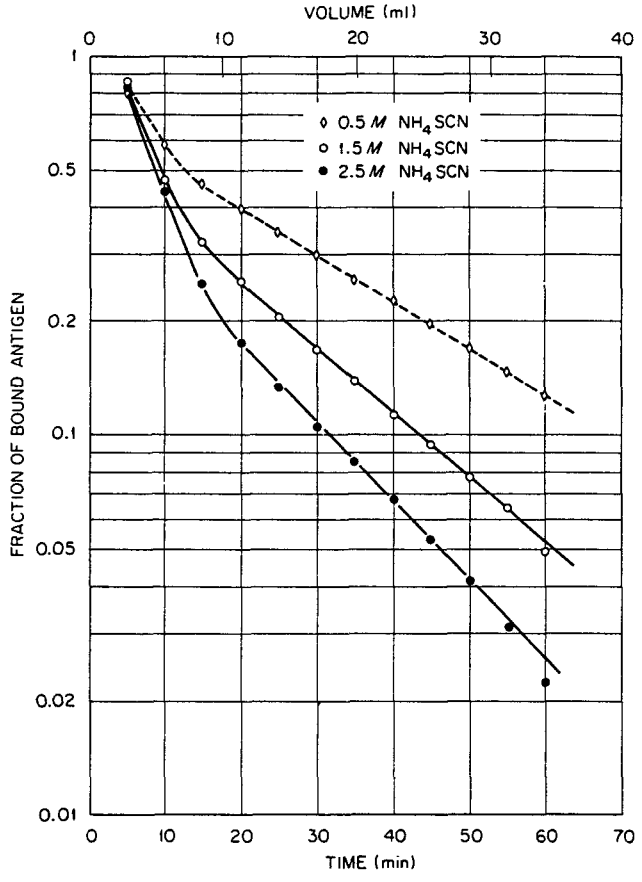

FIG. 6. Elution of antigen from a 5-ml Sepharose immunosorbent column as a function of time and eluent volume.

wash, and the overall effect of these small elution volumes is an apparent reduction in column capacity.

The effects of cycling the immunosorbent are best exemplified by the results of a typical experiment shown in Table 5. A 5-ml Sepharose anti-HA immunosorbent prepared from goat IgG (batch 1) was cycled using a 2-mg/ml solution of ^{125}I-labeled albumin with the following program: Flow rate, 0.5 ml/min; albumin solution, 6 min (3 ml); buffer wash, 36 min (18 ml); 2.5 M NH_4SCN, 30 min (15 ml); final buffer wash, 30 min (15 ml).

These results clearly show some of the effects that could be predicted from the known characteristics of immunosorbents examined earlier. The poor recovery of bound antigen from the column on the first cycle can be partly explained by an insufficient volume of eluent to completely dissociate the complex. However, the recovery from the second cycle indicates that the 15 ml of NH_4SCN used is capable of dissociating 90% of the adsorbed albumin. The difference of approximately 600 μg antigen from that expected to be released on the first cycle represents an apparently irreversible reaction of antigen. This "first-cycle effect" has been observed with other supports and using a variety of immunological systems, and must be considered in defining immunosorbent capacities. The results further show that, after the primary cycle, the recovery of antigen approaches a steady value from which preparative yields can be predicted.

Under the conditions of this experiment is is also obvious that there is a gradual accumulation of albumin on the column as a result of restricting the amount of chaotropic agent. From the standpoint of efficiency, increasing the volume of eluent would provide only a minor improvement in yield at considerable additional expenditure of time. It is more effective to accept this minor incremental loss throughout much of the separation and recover this antigen by a final elution using a larger volume of chaotropic agent. Similarly, reprocessing of the unadsorbed fraction to remove traces of a

TABLE 5. **Effect of Cycling a 5-ml Column with Excess Antigen and Eluting with 15 ml of 2.5 M NH_4SCN**

Cycle	Amount applied (mg)	Amount bound (mg)	Amount eluted (mg)	Eluted/bound (%)	Amount retained (mg)
1	5.87	4.192	3.204	76.4	0.988
2	5.97	3.316	3.019	91.0	0.297
3	5.89	3.239	2.988	92.2	0.251
4	5.88	3.179	2.964	93.2	0.215
5	5.86	3.135	2.995	95.5	0.140
6	5.91	3.089	2.965	96.0	0.124

valuable antigen is more efficient than attempting complete removal during a single separation with its attendant stringent conditions. This recycling principle is particularly valid when purity is of prime importance.

Rapid Cycling Operation. The effect on reaction rate obtained by restricting the sample load, discussed earlier, indicates a potential approach to decreasing the cycling time of an affinity chromatography system. In a reagent *production* application, overall efficiency is defined in terms of yield per unit time or effort. Therefore optimization consists in obtaining the greatest output in the least time, even though it requires some sacrifice in other parameters such as column capacity or maximum use of immunosorbent.

Limiting reaction to the outer layers of a porous immunosorbent can be accomplished in a very simple manner by adopting a high flow rate. This, in effect, limits the time the sample is in contact and hence the applied sample load. The result of this restricted reaction should be that diffusional effects, as exemplified by the slow rate constants in equilibrium experiments, are considerably reduced, thus allowing more rapid cycling.

To investigate the apparent loss of capacity resulting from an increase in flow rate, a column was prepared from 50 ml of HA–Sepharose 4B immunosorbent (containing a total of ~100 mg HA). To reduce the back-pressure at the increased flow rates, a 5-cm-diameter column was used in preference to the conventional 2.5 cm. A large batch of goat antiserum was obtained with the aid of an Aminco blood cell separator (batch 6); it was spiked with ^{125}I-labeled IgG purified from the same antiserum. The applied sample was 10 ml of this antiserum diluted with an equal volume of phosphate buffer containing 0.5% (vol/vol) Tween 80. The reaction times with buffer wash and 2.5 M NH_4SCN were adjusted by visual inspection of the UV monitor to give approximately equal proportioning of the "unadsorbed" and "adsorbed" fractions for each flow rate. The total collected volume of "unadsorbed" fraction (i.e., first-buffer wash) was between 100 and 120 ml; of the eluent between 80 and 120 ml/cycle. The column was washed with 2.5 M NH_4SCN between experiments to elute any antibody remaining from a previous cycle.

The results of increasing flow rate on recovery of specific anti-HA IgG are shown in Table 6.

These results show an apparent loss of capacity as the flow rate is increased; however, it becomes insignificant when the yield per unit time is considered. One major disadvantage of these shorter cycle times is the large volume of eluent obtained that require further dialysis and concentration. To reduce this additional effort, and thereby increase overall production efficiency, simultaneous concentration and dialysis was incorporated into the conventional system. This was accomplished by means of a hollow-fiber

TABLE 6. **Effect of Increasing Column Flow Rate on Recovery of Eluted Fractions**

Experiment no.	Mean flow rate (ml/min)	Cycle time (min)	Mean "unabsorbed" fraction (total cpm)	Mean "adsorbed" fraction (total cpm)	"Adsorbed" recovery (% of total)	Yield (cpm/h)
1	1.88	120	75,500	210,070	73.6	105,000
2	3.55	65	84,300	180,000	68.1	166,100
3	6.50	40	84,200	168,700	66.6	253,000
4	7.60	37	82,660	165,100	66.6	267,700
5	8.90	33	80,370	163,200	67.0	296,700

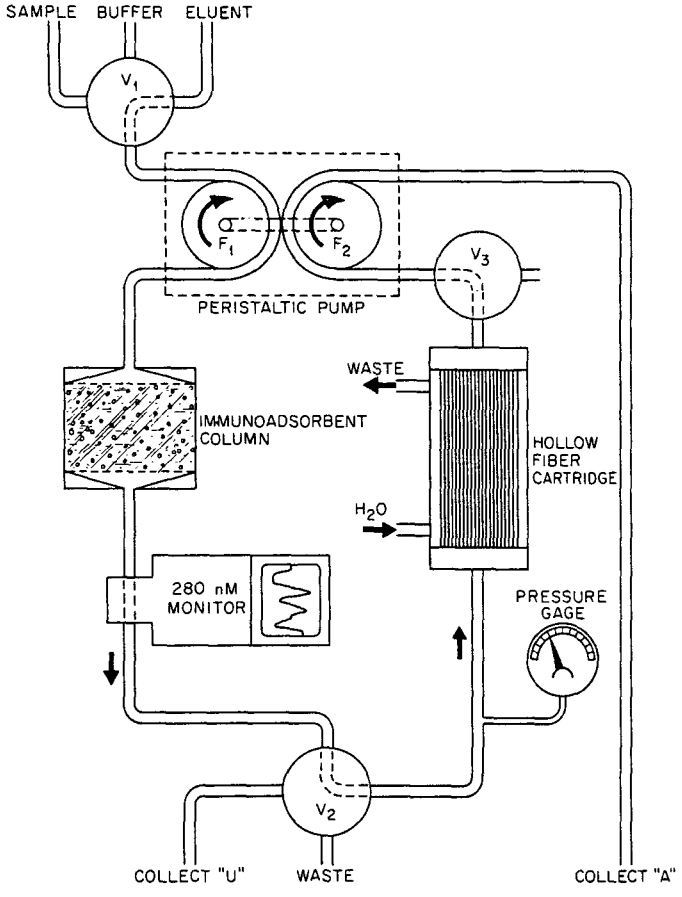

FIG. 7. Schematic diagram of continuous dialysis and concentration system used in rapid-cycling experiments.

TABLE 7. **Program for Rapid-Cycling Experiment**

Time (min)	Operation
0	Clock starts
0.5	Adsorbed fraction off
1.00	Sample on
4.00	Sample off
4.25	Unadsorbed fraction collect
15.50	2.5 M NH_4SCN eluent on
17.50	Unadsorbed fraction off
18.00	Adsorbed fraction collect
30.00	2.5 M NH_4SCN eluent off
37.50	Repeat

"miniplant" (BioRad Laboratories, Rockville Center, New York) placed in the "adsorbed" fraction output as shown schematically in Fig. 7.

The column effluent is pressurized in the hollow-fiber cartridge with the aid of a second channel of the peristaltic pump. This channel has a smaller tubing diameter than that in the primary column channel and, because it is connected to the cartridge output, creates a backpressure within the hollow fibers. The relative sizes of the pump tubes are chosen so that a pressure not exceeding 8 psi was developed, giving a concentration factor of about 4 : 1 of

TABLE 8. **Recovery of Specific Antibodies from Rapid (37 min) and Slow (150 min) Cycling Experiments**

Cycle no.	"Unadsorbed" fraction (total cpm)	"Adsorbed" fraction (total cpm)	"Adsorbed" recovery (% of total)
Rapid cycle			
1	79,292	158,536	66.6
2	92,000	176,505	65.7
3	95,864	175,160	64.6
4	93,656	172,368	64.8
5	95,580	168,838	63.8
6	97,278	165,522	63.0
7	102,742	171,258	62.5
Slow cycle			
1	61,698	144,060	70.0
2	76,320	158,875	67.5
3	81,782	165,984	67.0

the eluent. The recycling systems (2,3) are modified by incorporation of a positive output valve (V_2, Fig. 7) and a cartridge shutoff valve (V_3, Fig. 7); the latter is to prevent continuous pumping of the cartridge during other times of the cycle.

To assess the production efficiency of fast cycling, the program listed in Table 7 was used.

The mean value of the flow rate was 7.08 ml/min; the sample was that used for the preliminary recovery experiments, i.e., a 1 : 1 dilution of goat antiserum (batch 6) in phosphate buffer containing 0.5% Tween 80. The same level of spiking with ^{125}I-labeled IgG was used. The results obtained by counting the fractions from the first seven cycles (without dialysis or concentration of the eluent) are given in Table 8. Included in this table for direct comparison are the first three cycles of an identical system using a flow rate of ~2 ml/min and a total cycle time of 150 min.

The rapid cycling was continued for 20 additional cycles with continuous dialysis and concentration. The eluted fractions were combined and further concentrated to yield a total of 3240 mg of specific anti-HA IgG for a total cycling time of about 18 h.

DISCUSSION

The ease of preparing immunosorbents for preparative applications using CNBr-activated Sepharose provided a definite advantage over alternative chemistries and supports. However, the choice of this method and the inherent nature of the support material impose certain restrictions on its efficient utilization. The results obtained in this investigation have indicated the nature and, in some cases, the magnitude of these operational limitations that can be utilized in the design and manipulation of a practical system.

Although the reported findings were obtained using model antibody–antigen reactions, it is to be expected that other immunological systems will behave in a similar manner. This is especially valid for the capacity and dynamic studies using antibody-linked immunosorbents, since immunoglobulins can be considered as essentially chemically identical. In applying these findings to antigens other than the one used, however, consideration must be given to differences in molecular size and chemical and immunological reactivity. Similarly, the affinity of antibodies prepared in a given animal can vary with time of collection as well as being highly dependent on the immunization schedule. For these reasons each antibody–antigen system must be considered quantitatively unique. Generalizations can be made only from aspects that result from chemical and physical characteristics of the support material and system operational variables.

To amplify this important distinction, let us consider the finding that immunological reactivity is dependent on the amount of antibody covalently linked to the support. The results in Fig. 1 clearly show that the amount of CNBr used in the activation directly determines the amount of antibody covalently linked to the support. This can be considered a general finding applicable to any immunoglobulin preparation using Sepharose 4B and the conditions stated. A maximal binding of 20–25 mg IgG/ml of packed support can be predicted for any chosen antibody. This upper limit is probably determined by the number and accessibility of reactive sites and steric limitations imposed by the ~160,000 molecular weight immunoglobulin molecule.

However, the subsequent immunological reactivity of this linked antibody is highly dependent on the particular system under investigation. The values obtained in specific activity measurements will depend entirely on the amount of specific antibody in the immunoglobulin preparation, steric factors involving the size and reactivity of the antigen, and the experimental conditions being used. Therefore, in assessing the effects of any variable, such as the amount of linked reagent on the specific reactivity, only relative effects are meaningful. Absolute values are relevant only to the individual reagents used in a given system.

The theoretical maximum specific activity of a pure divalent antibody is 2.0 when expressed as the molar ratio. However, it can be expected that some covalent linking will involve portions of the molecule containing the immunodeterminant sites and hence will lower this maximal value. The finding that increasing the amount of linked antibody has a detrimental effect on its subsequent reactivity indicates that additional effects are involved. It is highly probable that these are due to steric hindrance or accessibility restrictions on the amount of antigen that react with an increasing density of antibody molecules. With highly porous supports it can be postulated that at high antibody densities many of the molecules themselves may severely restrict access of the antigen into the deeper pores of the matrix. Solid glass supports show a lower degree of protein density effects, since access to the reactive sites can be considered relatively unhindered. Further constraints on achieving maximum activity are provided by the strong possibility that the antibody distribution over the surface of the support may not be uniform or that the close proximity of a reacted antigen hinders access to adjacent antibodies. Since many protein antigens can be considered multivalent, there is a further potential of multiple attachments at high antibody concentrations, thus effectively reducing the overall reactivity of the immunosorbent.

The practical effect of this reduced reactivity is significant in the application of biospecific adsorption in that maximal utilization of an antibody preparation will be obtained only at low coupling densities.

However, the subsequent loss in capacity of a given volume of immunosorbent will require increased processing time and effort in preparative application. Therefore, the first of several compromises has to be accepted in designing an operational protocol to utilize the immunosorbent efficiently. If the amount of specific antibody is severely limited and maximum reactivity is essential, then it appears that some sacrifice in volumetric capacity must be accepted. Conversely, if the supply of reagent is not limiting, considerable saving of operation time and effort becomes possible by judicious utilization of highly loaded immunosorbents.

The results given in Table 1 on the effect of increasing substitution on reactivity also indicate that the method of coupling the antibody to the activated support affects subsequently immunological reactivity. It appears for the albumin system that an increase in reactivity is obtained by limiting the amount of activation rather than the quantity of protein available for coupling. This finding is compatible with the postulate that the overriding reactivity effect is dependent on antibody density or distribution. From an operational viewpoint, as will be discussed later, it would be preferable to restrict reagent to the more accessible surface layers of the immunosorbent bead. Limitation of the amount of protein in the coupling reaction offers a potential method of achieving this; however, it appears that some reduction in overall reactivity will have to be accepted if this approach is adopted.

Determination by means of equilibrium experiments of the affinity constant of antibodies allows prediction of separation efficiencies in the application of immunosorbents. This is demonstrated in Tables 1 and 2 for the particular sample of anti-HA used in the affinity constant determinations described previously. The predicted uptake and subsequent elution recoveries can be somewhat surprising if immunological reactions are naively considered to be irreversible. In employing affinity chromatographic techniques, the equilibrium constant is the fundamental characteristic that will ultimately determine efficiency in terms of purity, yield, and capabilities of the system.

Although the figures quoted are for an immunosorbent of somewhat low affinity ($\sim 3 \times 10^7$) and many antibody preparations have constants two or three orders of magnitude greater, there are several general conclusions to be drawn from these results. The first is that adsorption efficiency is directly related to antigen concentration. The higher the antigen concentration in the applied sample, the more efficiently it will be extracted. Of course, there is a limitation on the total amount applied, which is determined by the capacity of the immunosorbent, but this generalization applies equally at all levels of loading.

This observation has particular significance in immunosubtractive applications where the removal of a particular protein is desired. Irrespective of the initial concentration of the soluble species, some finite amount

will not be absorbed, dependent on the affinity of the immobilized reagent. Since it is conventional practice to follow sample application with a buffer wash, some desorption will occur at these effectively low concentrations, leading to further contamination. The amount of active species present in the unabsorbed fraction in many applications involving multiple antigen subtraction is minor compared with the contamination resulting from an "unbalanced" immunosorbent containing a nonstoichiometric ratio of antibodies (1). Both in immunosubtractive applications and in general preparative techniques where a high degree of purity is demanded, reprocessing the product is often a more efficient approach than attempting the process in one separative step.

Elution of the reacted species with chaotropic solutions is also affected by the affinity constant of the immunosorbent. As can be seen in Table 3, elution efficiency is directly related to the total volume of eluent applied (or, more precisely, to the concentration of eluted species in the eluent). It therefore appears desirable for preparative application to utilize lower affinity antibodies to reduce eluent volume and increase yield. Obviously, therefore, a compromise between adsorption and elution efficiencies must be accepted in the application of the method for preparative purposes. In a repetitive cycling system the effect of incomplete desorption results in an apparent loss in column capacity with successive cycles, as indicated in Table 5. After several cycles, using a fixed volume of eluent, the system stabilizes from the aspect of amount recovered per cycle. However the total amount is considerably less than that which is possible using larger volumes of eluent.

The first cycle of an affinity chromatographic system invariably has atypical adsorption and elution efficiencies. Although determined largely by volumetric considerations, this effect may have a more fundamental origin. It is more pronounced using antibody-linked immunosorbents, suggesting perhaps subtle modification of some of the molecules, resulting in an apparently irreversible reaction with antigen. It is therefore unwise to predict subsequent operation of a particular immunosorbent from the results of its first exposure to antigen, particularly in preparative applications.

Nonspecific reaction of reagents is highly dependent on the system under investigation. However, it is to be expected generally in the use of antigen linked supports to prepare monospecific antibodies. The beneficial use of nonionic detergents in reducing the problem is evident from Table 4. A possible explanation of the effect of the detergent is that it masks potentially reactive hydrophobic regions of the support or of the bound protein. The effect may be more fundamental, however, in that the detergent may effectively reduce denaturation of proteins as suggested by Boyer et al. (16). In practice, it is often preferable to remove unavoidable

contaminants in the eluted product by additional purification utilizing another chromatographic method. For example, the trace of albumin often present in specific antibody preparations, presumably due to nonspecific adsorption to the basic immunoglobulins, is more efficiently removed by simple passage of the material over DEAE-cellulose than by reprocessing over the antigen immunosorbent.

The porous nature of the matrix imposes the greatest operational compromises to be accepted in using immunosorbents based on Sepharose or porous glass. Since accessibility to the linked protein is determined by diffusional considerations, the observed reaction rates are undesirably slow for large molecules. Maximum utilization of the reagent necessitates very low flow rates in affinity chromatography or long incubations in a batch process. This is amply demonstrated in Fig. 4, which relates extent of reaction to time, or in Fig. 6, which relates extent of reaction to the volume of eluent from a small immunosorbent column. Because the rate of diffusion is an intrinsic property of the protein, dependent on its molecular dimensions, improvements can be achieved only by physical or operational means.

The use of nonporous supports has obvious advantages in reducing the effects of diffusion within the structure of the immunosorbent bead. The overall reaction rate becomes dependent only on the immunochemical rate and mixing within the interstices of the particles. This allows more rapid operation of solid support columns, with a high efficiency of both adsorption and desorption and minimal volume of buffer washes. The rigidity of the support provides excellent flow characteristics at high flow rates, making their use for specialized applications an attractive alternative. The major disadvantage for routine separations is the relatively low capacity of nonporous supports—an order of magnitude less than that possible with porous supports.

An alternative approach to improving the operation of porous affinity columns in preparative applications is offered by restricting reaction to the outer layers of the immunosorbent. This is most readily achieved by employing higher flow rates in the system. Although, as exemplified in Table 6, this may result in lower single-cycle recoveries, the savings in time are considerable. The major limitation to this approach is residual diffusion of unabsorbed components into and out of the porous bead during the buffer wash following sample application. Sufficient time must be expended to ensure removal of low molecular weight components from the columns; otherwise contamination of the desorbed protein will occur. The efficiency of elution is somewhat improved at the higher flow rates owing to the higher volumetric throughput. Any reactive protein that diffuses into the bead during desorption tends to become readsorbed during the subsequent buffer wash, thus contributing to the preponderance of surface reaction in the

following cycle. The disadvantage of increased volumetric throughput with the rapid-cycling system can be largely obviated by continuous concentration during dialysis as described earlier.

From a consideration of the results and discussion presented in this report, it becomes apparent that no completely general criterion for optimization of operating protocols is possible. Preparative efficiency can be defined only in terms of the availability of reagents and the purpose of the separation. Within this context, however, some generalizations can be discerned that allow a more rational approach to the practical utilization of immunosorbents.

Availability of material can be arbitrarily considered as being of four classes:

1. Unrestricted immobilized reagent and unrestricted supply of sample.
2. Unrestricted immobilized component and restricted supply of sample.
3. Restricted supply of immobilized species and unrestricted sample.
4. Restricted supply of both immobilized and sample components.

Each of these possible combinations dictates that different criteria be used in preparing and applying the method. Obviously, the first classification requires the least stringent conditions, for a relatively high degree of inefficiency can be tolerated throughout. In the second class, however, although latitude can be exercised in preparing the immunosorbent, operating conditions must be optimized to give a high degree of adsorption and recovery of the active species in the sample. To save time and effort, the availability of reagents in the third class demands that a low-protein-density, high-efficiency immunosorbent be prepared and that operating conditions be chosen that will provide maximal utilization of the immunosorbent potential disregarding a possible wastage of sample. The last combination offers the greatest challenge in optimization requiring efficient operation throughout and consideration of the more sophisticated methodologies mentioned in this report.

Although the availability of the reagents can influence the choice of procedures to be used, the ultimate objective of the purification further dictates the experimental approach. Again, these requirements can be broadly classified in terms of the quality of the product, the required total amount, the time available, and the facilities for automatic operation and additional processing. The danger of these simplified classifications is that unrealistic specifications are anticipated and may become impractical to achieve in many immunological systems. For example, the purity of the product does not depend only on a chosen plan of optimization experiments, but is primarily dependent on the original purity of the immobilized reagent. However, within these restrictions the proper choice of the available variables invariably offers considerable improvements over the empirically

selected intuitive approach. High purity in a product often demands that yield and operational time be sacrificed in removing the last traces of contaminants. Conversely, a high yield can be readily obtained, as demonstrated by the rapid-cycling procedure reported, provided that quality is somewhat compromised.

Finally, the practical advantages offered by reprocessing an eluted product should be considered before investing time and effort into experimentally finding acceptable compromises in a single separation. Quite often the purity of a product can be markedly improved by the simple expedient of repeating the separation using identical conditions. However, a second separation using different operational variables can often provide a more acceptable product with a significant reduction in total effort and separation time. Similarly, the combination of the biospecific adsorption techniques with a separation method based on other principles, such as ion exchange or gel filtration chromatography, can be invaluable in many difficult or specialized preparative applications.

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