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Analytical Techniques for Cell Fractions

XX. Cyclic Affinity Chromatography: Principles and Applications

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Principles and applications of an automatic cyclic affinity chromatography system, especially as applied to separation of proteins by immunosorption, are described. Use is made of columns of either immobilized antigens or antibodies to separate from a mixture, in a repetitive fashion, a desired protein or proteins by immunoadsorption followed by elution or to take from a mixture all proteins but the desired ones by allowing these latter to go through unadsorbed. The amplification provided by the cyclic use of the system and the biological amplification of hyperimmunization in achieving useful yields of desired proteins is discussed. Experience in the use of eluting solutions which do not cause damage to either the fixed or eluted proteins is presented. Representative separations done with the system are described, illustrating the different modes of use.

The objective of this paper is to outline general separation methods based on affinity chromatography which are applicable to the fractionation of complex mixtures and to the problem of isolating minor constituents including tumor-associated autoantigens from tissues, plasma, tissue-culture fluids, or urine. The methods depend on amplification provided by the cyclic use of affinity columns and on the inherent amplification of the immune systems of immunized experimental an-

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imals. In addition to separations based on the selective adsorption of desired moieties, immunosubtraction offers the unique possibility of isolating labile proteins by contaminant subtraction, i.e., by binding all undesired proteins to a column while the desired protein passes through.

In the preceding paper (1) the instrumentation required for cyclic affinity chromatography was described. In this paper we present a review of different approaches to protein fractionation based on the use of stereo-specific adsorbents, together with methods of adsorption and elution. The elements of all the separations have been previously described but have not been integrated conceptually and experimentally. We have presented preliminary discussions of some of the work reported here (2,3).

SEPARATIONS METHODS

The use of affinity chromatography to purify antibodies or antigens once either is available in a pure state is well known (4). In addition columns of normal serum proteins have been used to subtract antibodies to them from antisera against hepatitis-associated antigen (5). Here we attempt to organize fractionation methods based on immunoadsorption in a systematic manner, with emphasis on the amplification.

Bootstrapping. We have used the term "bootstrapping" to describe a series of steps using both the amplification inherent in the immune systems of higher vertebrates and the amplification made possible by cyclic affinity chromatography. The use of this technique is illustrated diagrammatically in Fig. 1. In this and all subsequent diagrams Cyclum systems are illustrated diagrammatically as a column with two attached squares below, the first of which indicates a uv monitor attached to a strip-chart recorder, while the second and lower square indicates the distributor valve which passes unadsorbed protein (the "U" fraction) to a collecting vessel on the left and adsorbed and then eluted protein (or "A" fraction) to the vessel on the right. On all charts the unadsorbed peak ("U" fraction) is shaded for ease in identification.

Given a very small amount of a pure antigen, it may be used to produce an antiserum (Fig. 1, step 1), often of rather low titer. The IgG (immunoglobulin G) from this antiserum (step 2) is then isolated and used to prepare a first-generation affinity column. By prolonged recycling of this column, and by pooling and concentrating all the "A" fractions, sufficient antigen (step 3) may be prepared to allow preparation of much larger amounts of antisera (step 4) and a column of immobilized antigen. With this combination rather large amounts of *monospecific* IgG may be prepared (step 5). This in turn may be used to prepare a very large second-generation IgG column for larger-scale antigen production (steps 6 and 7). Many variations of this procedure are pos-

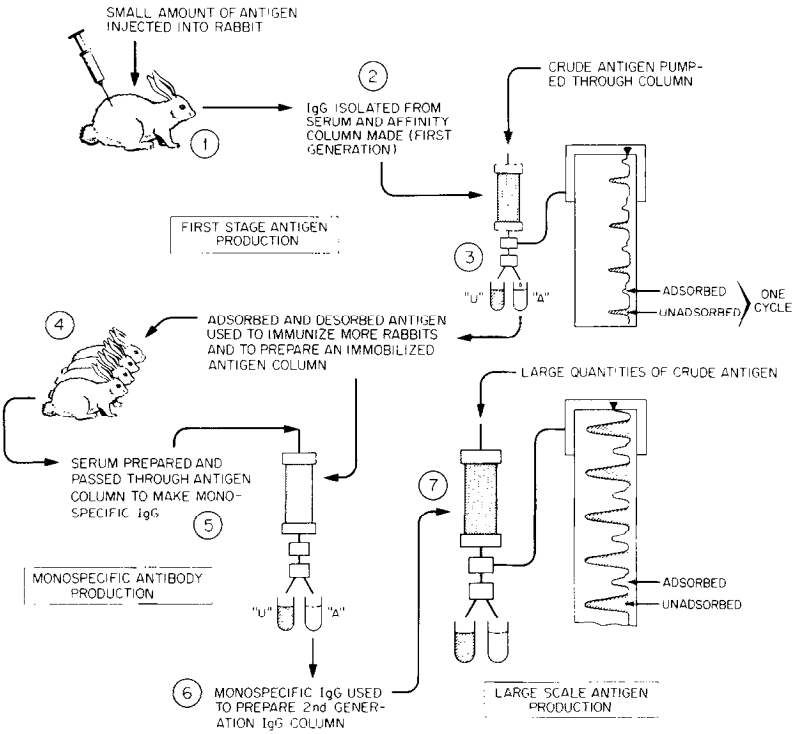


FIG. 1. Bootstrapping technique.

sible: for example, one may start with antisera or antigen or antibody eluted from a complex, as may occur in the kidney. As discussed subsequently, problems with contaminating antigen will occur and may require introduction of additional steps, and additional stages of amplification may also be required. To be really effective an amplification of at least 100 should be possible at each stage.

All-but-one subtraction. With very labile antigens methods are required that are rapid and involve minimum manipulation. Surprisingly such antigens are often quite stable if linked to an insoluble support. A general method for isolating labile antigens is shown diagrammatically in Fig. 2 and depends on the preparation of a column that will remove everything from a sample except the antigen desired.

Initially (Fig. 2, step 1) the antigen of interest is isolated by conventional means and immobilized (step 2). At the same time rabbits or goats are hyperimmunized against the starting antigen mixture (step 3). (Techniques for producing balanced antisera against mixtures are described in a later section.) The harvested serum (step 4) is passed over a column of immobilized starting antigen mixture (prepared in step 5) to prepare

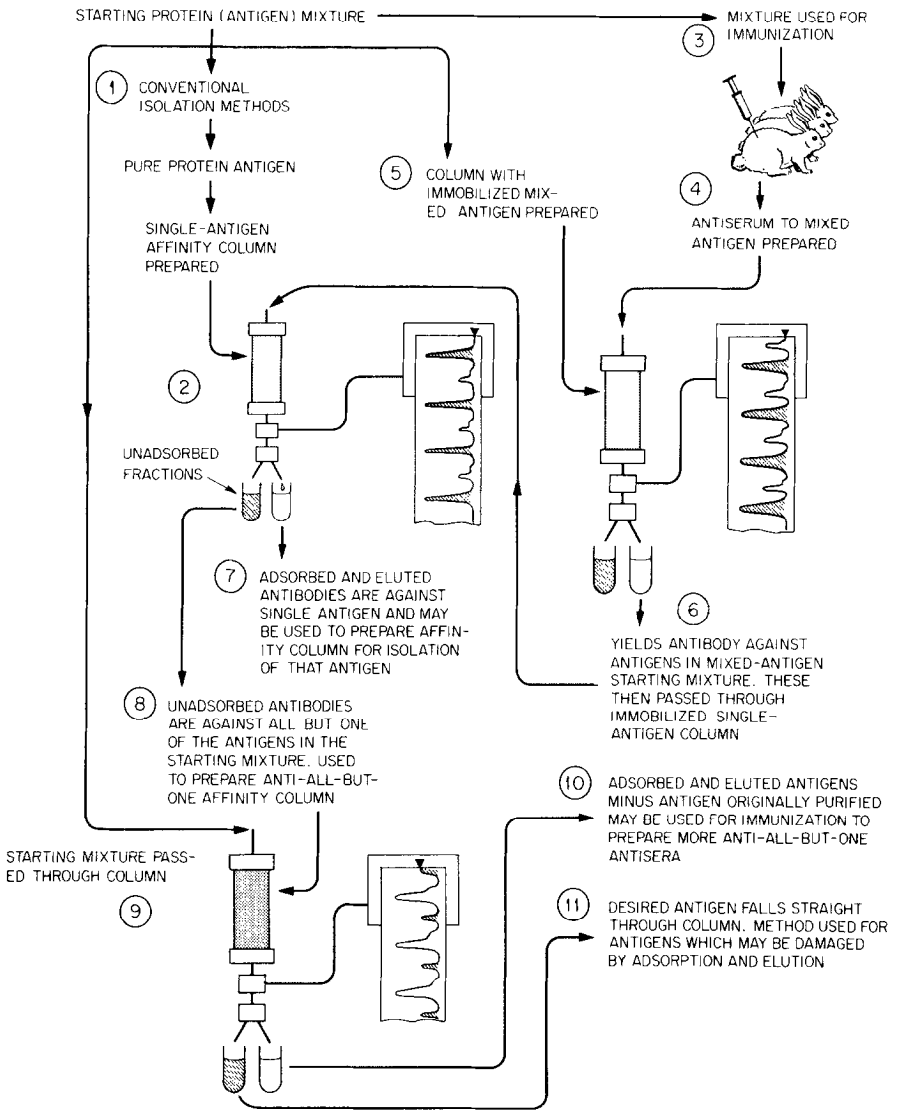


FIG. 2. All-but-one subtraction.

purified antibodies against it (step 6). When these antibodies in turn are passed over the single-antigen column, the antibodies against that antigen are adsorbed (step 7), while the unadsorbed antibodies pass through (step 8). The latter, which include antibodies against all but one of the antigens in the initial mixture, are now made into a much larger column (step 9). This may now be used for preparing the single antigen of interest by cyclic application of the starting mixture, in which case the

desired antigen falls directly through the column. The adsorbed antigen, after elution, may be used to prepare even larger antigen columns for the preparation of more anti-all-but-one antiserum.

This general method will probably find greatest use in the preparation of serum lipoproteins and labile enzymes.

Frontal separations. Immune systems rarely if ever respond stoichiometrically to all antigens in a mixture used for immunization; in fact the immunogenicity of different proteins varies enormously. This simple fact may be made the basis of a general separations method termed *frontal separation* which is illustrated in Fig. 3. Antisera are prepared against a mixture (Fig. 3, step 1) and the IgG isolated and used to prepare an affinity column (steps 2 and 3). If a dilute solution of the starting antigen is pumped continuously through the column (step 4), it will saturate along its length at different rates for different antigens depending on the ratio

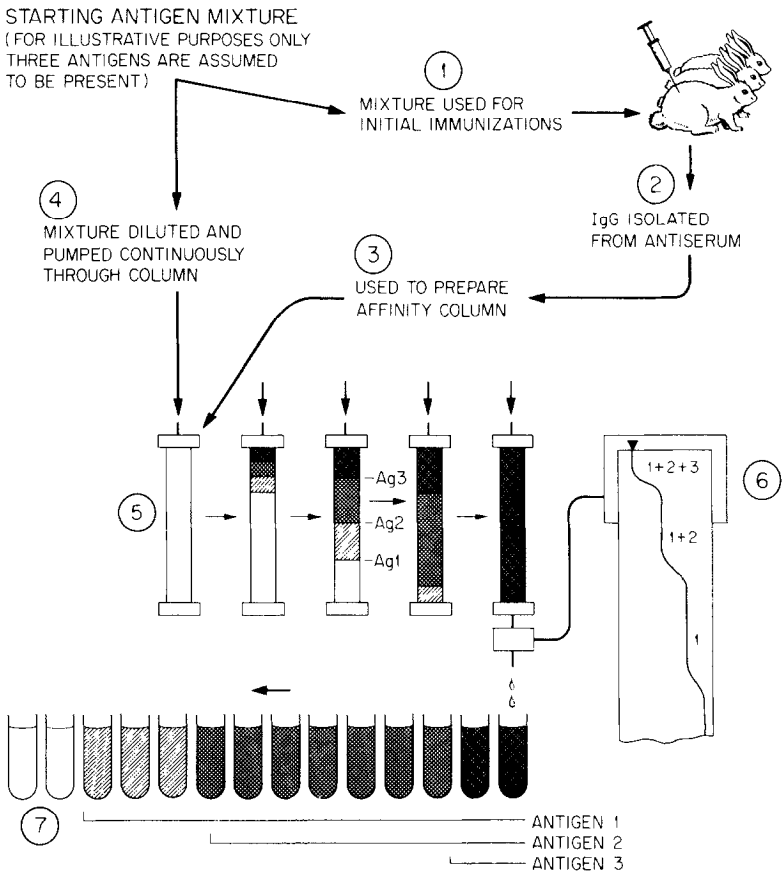


FIG. 3. Frontal separation of antigens on an unbalanced antibody column.

All our samples are routinely counted to an accuracy such that $2\sigma \not> 0.5\%\ \bar{x}$. All cpm are converted to dpm using the AES ratio method, and a standard curve freshly constructed for each experiment.

(*g*) *Calculation of Results*

A small amount of background radioactivity persists in the sample even after purification stages, and this becomes more significant the lower the levels of prostaglandins to be measured. For this reason we recommend that a "blank" sample be run in every series, and that the background radioactivity in each of the two zones measured.

Disintegrations per minute in the samples is therefore calculated by subtracting the background thus:

Δ dpm = dpm in sample − dpm in blank (background) and;

$$\mu \text{ units of prostaglandins in unknown sample} = \frac{\Delta\text{dpm in } F_{2\alpha-\beta} \text{ zone}}{\Delta\text{dpm in Me-}F_{2\alpha-\beta} \text{ zone}} \times \mu \text{ units of internal standard added,}$$

where "units" of prostaglandins are milligrams or millimoles, etc.

(*h*) *Preliminary Purification of the Sample*

Unless the known sample contains only the prostaglandins to be assayed, it is important that some purification procedure is adopted. The chief object of this is to separate prostaglandins from other lipid soluble reducible substances which would interfere with the assay.

In practice such procedures need not be elaborate: a variety of suitable extraction techniques are discussed in a review by Shaw and Ramwell (ref. 10) to which the reader is referred for further information. At least one of the purification procedures should include a thin layer, or column chromatography step. It is particularly important to ensure that E-type prostaglandins are separated from other prostaglandins which give rise to the same or similar reduction products ($F_{2\alpha-\beta}$) especially 15-keto E or F compounds and PGD compounds. If necessary PGE_1 and PGE_2 must be separated by argentation chromatography, (38).

(*i*) *Sensitivity of the Assay*

Using pure E-type prostaglandins, sample sizes as low as 1 ng ($\simeq$ 2.8 pmoles) can be quantitated; in determinations at this level, however, the difference in radioactivity between the "blank" and the sample to be assayed is of the order of 10^3 cpm (i.e., about 5×10^3 dpm); hence "1 ng" should be regarded as being almost the limit of the assay sensitivity. Because losses invariably occur during sample purification procedures, however, we do not recommend that this assay is used for *samples of biological origin* containing 10 ng ($\simeq$ 28 pmoles) or less of E-type prostaglandins.

factory method of evaluating an antiserum for this purpose is to actually use it to make a column and then determine its subtractive characteristics.

It might be thought that an unbalanced antiserum could easily give rise to a balanced one by passing it over a column of immobilized starting antigen mixture, and then eluting the bound antibodies. However, again some antigens bind much more antibody than is required to remove them from solution because of their size and because of the number of antigenic sites they bear. Thus adjustment of the proportions is still needed.

Two general procedures for doing this are outlined in Fig. 4. In the first (Fig. 4A) the initial step is the preparation of antisera against the mixture (step 1), and all of the antibodies thus produced are recovered on a column of immobilized antigen mixture (steps 2 and 3). The specific IgG thus isolated (step 4) is used to prepare an affinity column (step 5). The saturation profile of this column is then determined (steps 6 and 7) to discover how unbalanced it is and what sample volume should be used to isolate the antigens which quickly saturate the column (i.e., break through first). Those antigens are then isolated by repeating cycling and used as an antigen to boost the rabbits or goats used for antiserum production (steps 8 and 9). The antisera thus obtained are used to prepare a second set of columns which are in turn used to prepare additional boosting antigen (second-round antigen)—a process continued until the animals used are making an antiserum that will yield a balanced column (step 10). In many cases a mixture such as serum contains two major antigens (albumin and IgG) and many minor ones. Specific antisera against albumin and against IgG may be separately prepared and used to prepare batches of adsorbent which may then be mixed to give the proper ratio to remove both proteins completely from a known volume of serum. The fall-through from such columns may then be used to immunize animals using the scheme given in Fig. 4A.

In the procedure shown in Fig. 4B no attempt is made to prepare one column or antiserum that is completely balanced with reference to a given mixture. Instead specific anti-mixture antibody is prepared (specific in the sense of not containing inactive IgG) as in Fig. 4A and used to prepare a large specific-antibody column (steps 1–4). The antigens either not bound or bound in insufficient quantity to this column when a given amount of the starting antigen mixture is applied are then isolated (step 5) and used to immunize a second round of animals (step 6). Antibodies from these animals are in turn isolated (steps 7 and 8) and are used to prepare a second antibody column (step 10). Second-round antigens for which this second-round column lacks capacity are then isolated (step 9) and used to prepare third-round antigens (step 11). This

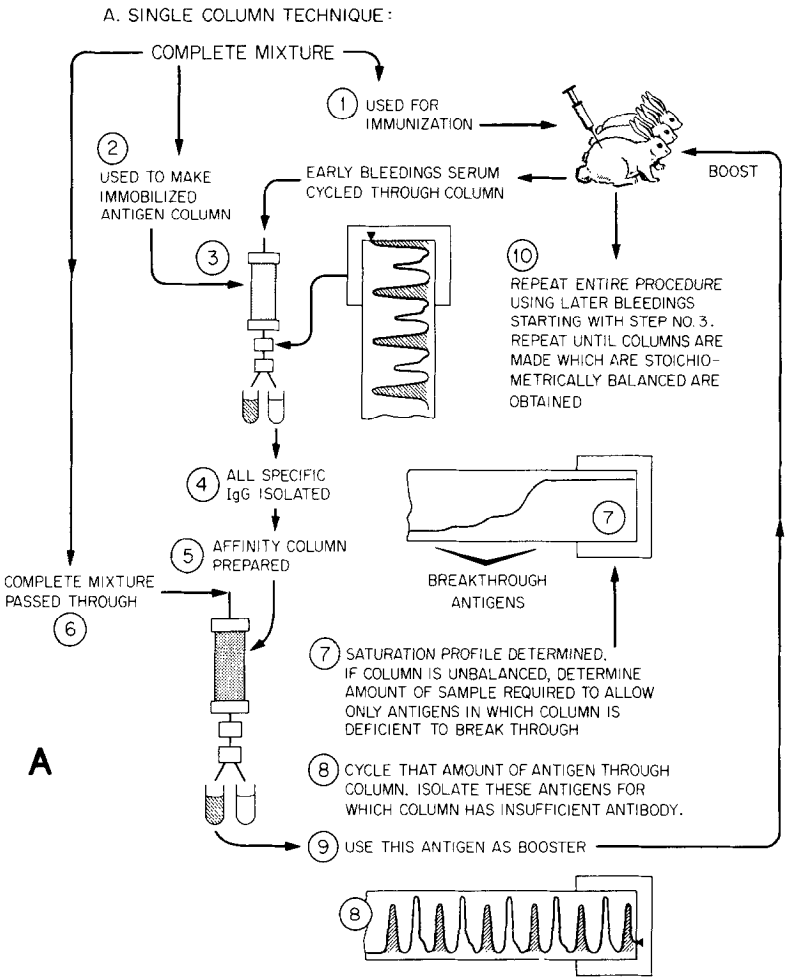
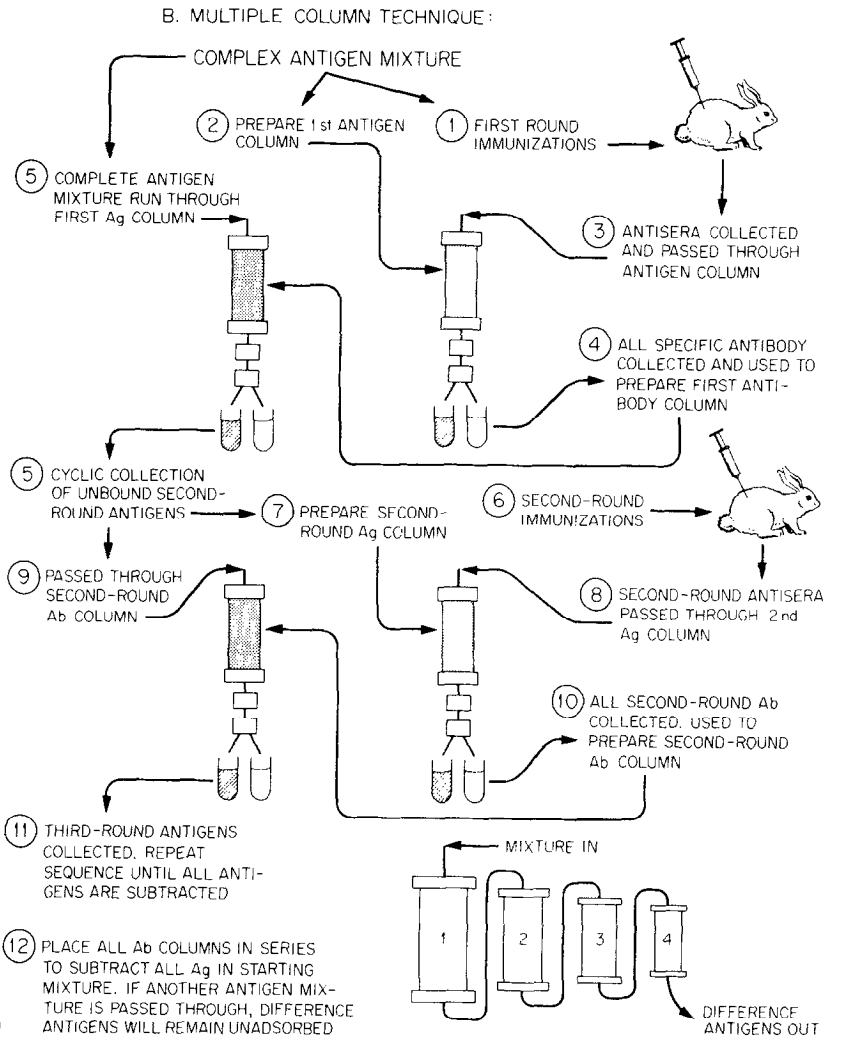


FIG. 4. Methods for preparing balanced immunosubtractive columns. A. Single column technique. B. Multiple column technique.

series is extended until all or nearly all antigens from the starting mixture are subtracted. All columns are then placed in series (step 12), their relative volumes adjusted, and used cyclinically to subtract mixtures for comparative purposes. For example, when all serum proteins of normal nonpregnant adults are subtracted from the sera of pregnant women, so-called pregnancy proteins remain. Alternatively all adsorbants may be blended in one column.

An additional use of columns balanced to match a mixture is to isolate a single species from that mixture when the best that can be done with other preparative methods is to enrich the starting mixture in that



species. For example if the concentration of antigen X is increased, by any procedure, to twice that in the starting mixture, an amount of starting sample may be found experimentally which will remove all the antigens including half of X, letting the other half pass through. With repeated cycling, and given relatively large amounts of the starting mixture, quite pure samples of X may be obtained and used to start the bootstrapping procedure.

Sandwich columns. Two kinds of sandwich columns have been explored. In both cases an antigen (or antibody) is covalently linked to the support, and then used to remove that species (not its opposite) from

solution. For example, serum albumin as a contaminant in a preparation can be removed in the following way. Serum albumin is covalently linked to a column support, and an antiserum containing antibodies against serum albumin passed over it. The column is then well washed, and the preparation containing the albumin contaminant passed over it. The contaminating albumin is then bound noncovalently to the antibody which is noncovalently bound to the antigen which *is* covalently attached. During column regeneration both of the outer two sandwich components are removed. This method is convenient only for small-scale work, since the middle component (which may be either antigen or antibody) is lost.

In the second type of column, developed by one of us (6), the antigen (or antibody) covalently linked to the column support is used to remove its opposite number from solution, the column is then washed, and the two moieties (antigen and antibody) are then covalently linked together with a reagent such as glutaraldehyde. This *reverses* the column from an antigen to an antibody one (or vice versa) and the column may then be used to subtract the species originally covalently attached to the support.

This does not by any means exhaust the possibilities opened up by the combination of stereospecific adsorbents and the amplification inherent in rapid recycling and in the immune system itself. In theory, with three or four stages of amplification, factors of 10^6 appear feasible. If gel filtration can also be adapted to relatively rapid cycling so that only mixtures of a narrow molecular weight range need be dealt with, then the problems of immunosubtractive reagent preparation can be greatly simplified.

In the preceding paper the basic instrumentation required for cyclic column operation was described. Thus far in this paper we have been concerned with principles applicable to such systems. We now ask, can they in fact be made to work, and what are their limitations?

INITIAL EXPERIMENTAL SYSTEM

A very large number of variables must be considered in developing procedures, and the problem of optimizing separations is extremely difficult to approach initially. At the outset therefore we have made the following decisions, based in part on a long series of orienting experiments.

Elution. The initial problem is the general class of eluting agent to be used, and four were considered. The first class employs extremes of pH (7). This is effective for many purposes; however, in our hands columns were gradually degraded after continuous use and also presented the problem of pH reequilibration (generally a time-consuming procedure)

before each new sample is applied. In addition many antigens are destroyed by high or low pH. An advantage of pH elution, however, is that neutralizing solutions may be pumped directly into the column effluent line, making exposure of an adsorbed and eluted moiety to pH extremes quite brief. The second class of eluents includes strong salts which are not specifically chaotropic. This method does not appear sufficiently universal for our purposes. The third class encompasses substances such as urea which tend to break hydrogen bonds and to disrupt secondary and tertiary protein structure. Our experience with urea indicates that it gradually damages affinity columns, denatures many of the substances eluted, is not an efficient eluent, and, in the concentrations which must be employed, is not easy to get rid of. In addition isomerization of urea may gradually occur in concentrated solutions, and a number of troublesome impurities may be present.

The fourth class includes chaotropic ions.[6] Of the commonly available anions, thiocyanate and trichloroacetate appear most effective, while lithium is one of the few useful cations. For introductory studies we have employed ammonium thiocyanate, and the effect of replacing ammonium with lithium will be reported later.

Temperature. Low temperature slows denaturation, adsorption, elution, and dialysis, possibly all to a comparable degree. We have therefore sought to operate all columns at room temperature and as rapidly as possible until a measurable advantage of working at lower temperatures is seen. Samples and collected fractions are refrigerated however.

pH. All solutions have been at or very close to pH 7.0 and have been buffered with 0.1 M sodium phosphate. The possible advantages of performing all steps at a different (but constant pH) require exploration.

Bacteriostasis. When protein separations systems are to be run for extended periods, it is essential to incorporate bacteriostatic agents in all solutions. Sodium azide, 0.1%, has been adopted as standard, and for protein samples it is convenient to make up a 20% solution and add one drop per 10 ml.

Monitoring. Thiocyanate and azide solutions have considerable absorbance at 260 nm but have very much less at 280 nm. Monitoring at the latter wavelength or at 285 or 290 nm is therefore used.

Flow rates. We have initially sought cycle times of 1–2 hr. This has necessitated flow rates ranging from 1 to 4 ml/cm^2/min.

[6] The word "chaotropic" was coined by Hamaguchi and Geiduschek in a study (8) of the effect of electrolytes on the stability of DNA secondary structure. Use of chaotropic ions for solubilization and dissociation of antigen–antibody complexes was extensively studied by Dandliker *et al.* (9), following earlier studies of Kleinschmidt and Boyer (10) on the effect of anions of the lyotropic series on antigen–antibody reactions.

INITIAL STUDIES

Effect of thiocyanate concentration on elution. The effect of various concentrations of ammonium thiocyanate on elution was examined by applying samples to an affinity column and then eluting successively first with one of a range of concentrations of thiocyanate and then with a high concentration to strip the column in preparation for the application of the next sample. The test system used was a Sepharose 4B column to which fetal calf serum proteins were covalently linked by activation with cyanogen bromide. The adsorbed moieties were antibodies in whole serum against fetal calf serum proteins prepared by hyperimmunizing rabbits. The results over a range of ammonium thiocyanate concentrations extending from 0.5–3.0 M are shown in Fig. 5, and the details of column operation are given in the figure legend. The volume of thiocyanate solution used in each elution event was ~50 ml. At the end of the experiment two additional pulses of 3 M thiocyanate were given to show that the column was indeed stripped so far as that concentration is concerned. As many as ten additional stripping pulses gave the same absorbance peak indicating (as was also shown by passing the eluting solution straight through the flow cell without passing through the column) that the absorbance seen is due to the eluting solution itself. While higher concentrations of thiocyanate may be useful for other systems, we conclude that 3 M is suitable for use with this test mixture.

The dialyzer water flow rate in this experiment was 0.33 liters/min, and this reduced the thiocyanate concentration, as determined refractometrically, to an average of 0.05 M in all the samples eluted with 3.0 M thiocyanate. The thiocyanate solutions absorb strongly in the ultraviolet with the absorbance rising very rapidly in the region of 280 nm. The absorbance recording shown in Fig. 5 was therefore made at 285 nm, and the flow cell was positioned downstream from the dialyzers so that most of the thiocyanate had been removed when the absorbance was determined.

Effect of change in sample size on amount bound. Having settled on 3 M thiocyanate for elution, the effect of changing the sample size was examined, as shown in Fig. 6, with experimental details again given in the figure legend. The amount of antibody bound increased almost linearly as the sample size was increased from 1–5 ml, as indicated by the height of the elution curves. However it must be stressed that we are working with antibodies against a mixture, and because more antibodies can be bound with increasing serum sample size does not mean that the column may not be already overloaded with one or more species of antibody against a very potent antigen. All the unbound effluents (U fractions) from the experiment in Fig. 6 were therefore pooled and concentrated, back to the total sample volume (15 ml), as was also the pooled

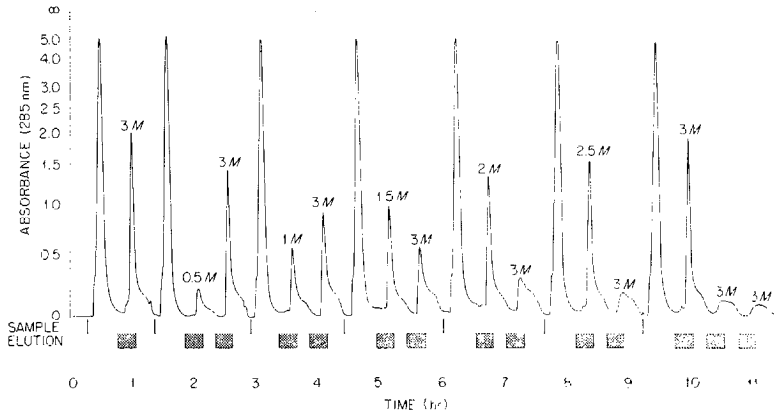


FIG. 5. Effect of thiocyanate concentration on elution of rabbit antibodies from fetal calf serum proteins covalently linked to Sepharose 4B. Two-milliliter samples of undialyzed rabbit antiserum were applied at times indicated followed by 100 ml of buffer. Sixty milliliters of one of a series of increasing concentrations of thiocyanate was then passed through the column as indicated on the chart, followed by 40 ml of buffer, and then 60 ml of 3 M thiocyanate. The column was then washed with 60 ml of buffer in preparation for the next sample application. In the first cycle the variable concentration of thiocyanate was omitted. At the end of the last cycle two additional 60-ml volumes of 3 M thiocyanate were passed through to indicate that no further protein could be eluted. Absorbance was measured at 285 nm using a 0.2-cm flow cell. The flow rate was 203 ml/hr.

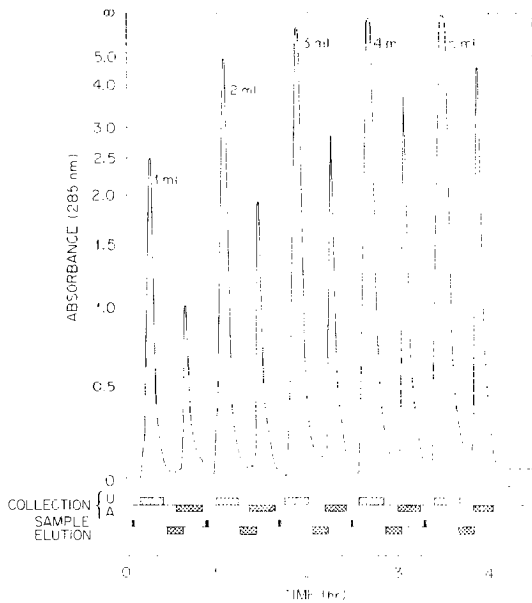


FIG. 6. Effect of sample size on amount of antibody bound to fetal calf serum column. The conditions of column operation were similar to those described for Fig. 5. All elutions were done with 3 M ammonium thiocyanate.

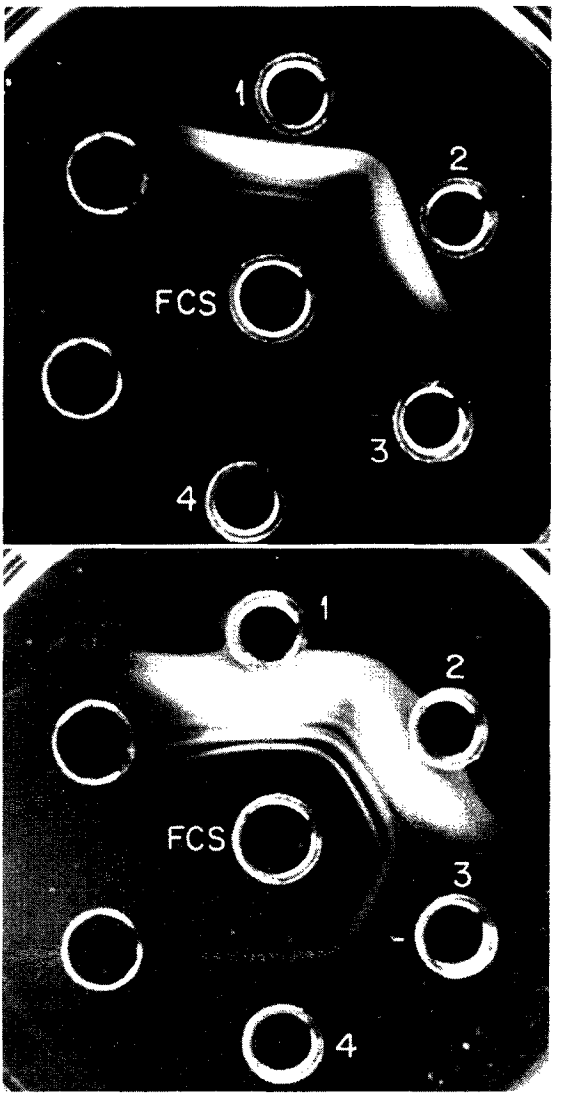


FIG. 7. Analysis of samples from the experiment shown in Fig. 6 to determine whether all antibodies had been removed from the starting serum. Unbound (U) and adsorbed and eluted (A) fractions were concentrated back to starting volume and examined by double diffusion on Ouchterlony plates. The center well contains fetal calf serum; well 1, starting rabbit anti-fetal calf serum; well 2, fraction A from Fig. 6; well 3, fraction U from Fig. 6; and well 4, fraction A from Fig. 8. Upper photograph at 24 hr, lower at 48 hr. Note that column failed to remove all of at least one antibody under the conditions employed.

adsorbed and eluted antibody (A fractions). These were tested for activity by double diffusion against fetal calf serum to see if all antibodies had been removed from the serum and whether the eluted antibodies were still functional. The results are shown in Fig. 7. It is evident that a spectrum of antibodies was removed from the serum by the column and that these were eluted in active form *but* that at least one antibody species was incompletely removed. The fact that that species exists in good concentration in the eluted antibodies suggests that it was not against an unstable antigen not found on the column. Rather it appears that it is against an antigen which is a very effective immunogen and that excess antibody is produced. Note that the principles described under frontal analysis apply here.

The completeness of removal of antibody from the unbound fraction

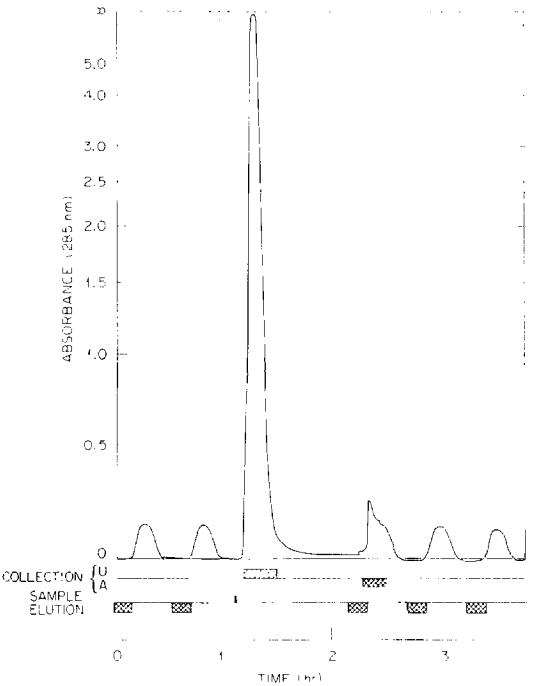


FIG. 8. Passage of unadsorbed protein from the experiment shown in Fig. 6. The proteins were reconcentrated to original volume and a 5-ml sample applied (300 mg of protein). Nearly all protein appeared in the unadsorbed peak. As expected from double-diffusion analysis, a very small adsorbed antibody peak is seen. In order to be able to distinguish thiocyanate absorption from protein in the eluted peak, ten cycles of thiocyanate 15 min long preceded sample application (of which the last two are shown), and two additional thiocyanate cycles were done after elution of bound protein. Column and flow conditions were the same as previously described. Analysis of the A peak from this experiment is given in Fig. 7.

was checked by passing the reconcentrated unbound proteins from the experiment shown in Fig. 6 back through the column. To obtain a good indication of the amount of protein bound, ten cycles of elution with 3 M thiocyanate were done, followed by sample application, followed by three cycles of 3 M thiocyanate elution as shown in Fig. 8. Subtracting the thiocyanate absorbance from the first post-sample elution peak indicates that only a very small amount of active antibody remained and was adsorbed. When concentrated, the A peak from Fig. 8 gave a line of identity on Ouchterlony plates with the antibody in the pooled U peaks from Fig. 6.

The reverse experiment—the demonstration that the antibodies bound in the experiment of Fig. 6 would bind again—is shown in Fig. 9 where only a very small fraction of the absorbance is seen in the unbound peak, the majority being eluted with the thiocyanate. In double-diffusion

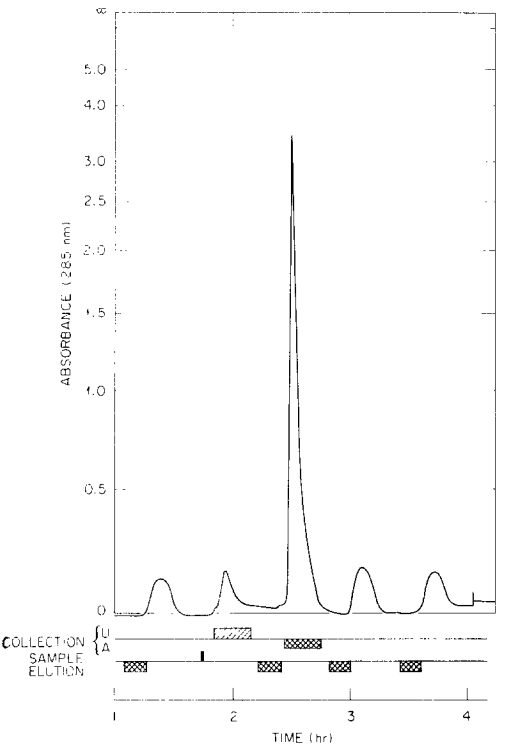


FIG. 9. Demonstration that protein adsorbed and desorbed from column of Fig. 6 will rebind to column. The combined A fractions were concentrated to the original volume of serum used, and 5 ml was applied to column. Only a small amount of unadsorbed protein is seen. One cycle of thiocyanate preceded sample application and two followed it to indicate absorbance due to thiocyanate and that the column was stripped. Flow rate, 229 ml/hr.

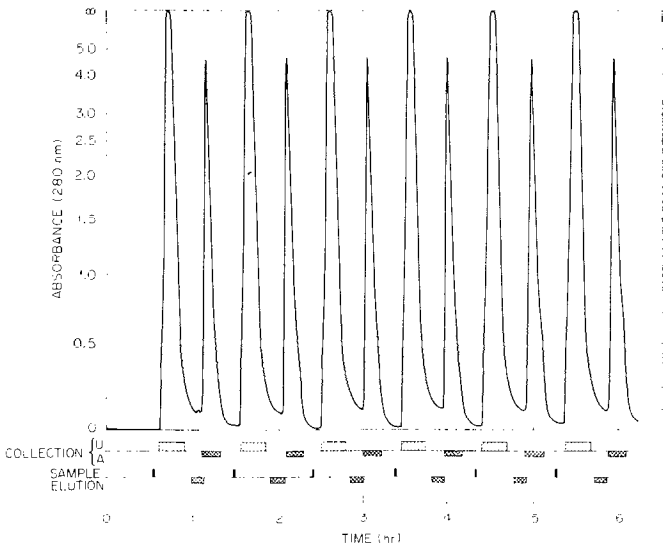


FIG. 10. Cyclic separation of antibodies to fetal calf serum protein from rabbit antiserum on column of immobilized fetal calf serum proteins. Five-milliliter samples were applied at intervals indicated, and elution was done with 3 M ammonium thiocyanate, in pH 7.0, 0.1 M sodium phosphate. Note exact reproducibility of elution peaks. Timing was as follows: Buffer, 20 min; sample, 1.6 min; buffer, 25 min; thiocyanate, 10 min. Total cycle time, 56.6 min; flow rate, 187 ml/hr.

experiments full activity by the antibody recovered from this experiment was observed.

These experiments do not answer the question of what the total capacity of the column for antibody is. This question is addressed later.

Repeated column cycling. The central problem is whether columns can be recycled repeatedly, and are the results precisely the same for first and subsequent cycles of a series? It might be thought that a population of antibodies of very high affinity might exist, which, although present in low concentration in a serum, would gradually saturate the column. The presence of such antibodies in an antibody column would have little effect since these would be quickly saturated, and the lower affinity antibodies would then continue to function. However with an antigen column such as the one used here, that would not be the case. Instead one would expect to see column capacity gradually decrease as saturation with postulated high-affinity antibodies occurred. The fetal calf serum column was therefore multiply cycled using 5-ml serum samples from the same serum pool used in previous experiments. The results for six cycles, each of 56.6 min is shown in Fig. 10. No detectable difference in the amount of antibody eluted can be seen in any of the six A peaks. In other experiments no noticeable decrease in capacity

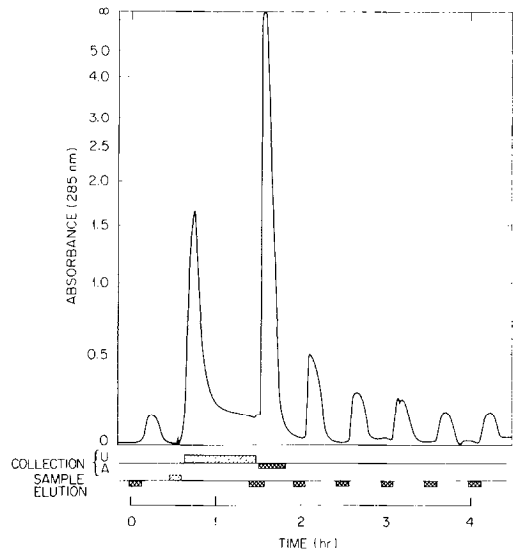


FIG. 11. Determination of capacity of immobilized fetal calf serum column for rabbit antibody. The remaining A fractions from the experiments shown in Figs. 5 and 6 were combined, concentrated, and passed through a fetal calf serum column. Protein, 295 mg, was recovered in the adsorbed and eluted A fraction. Sample, 26 ml, containing a total of 496 mg of protein, was applied. Since the column volume was 31.7 ml, the column capacity is at least 9 mg/ml. This experiment demonstrates the amount of antibody which can be bound and also raises the question of whether the U fraction observed here was not bound because the capacity of the column was exceeded or because of inactivity of the antibody. This question is examined in the experiment shown in Fig. 12.

has been noted in as many as 100 consecutive runs. This suggests that the amplification theoretically obtainable by recycling affinity chromatography can indeed be achieved in practice, although different detailed procedures may be required for quite different antigens.

Column capacity. Part of the antibody recovered from the experiments shown in Figs. 6 and 10 was passed over the fetal calf serum column (496 mg of protein in 26 ml) to determine the total capacity of the column. Note that in the runs in Figs. 6 and 10 the column was never saturated and that the antibody recovered could therefore be very markedly imbalanced with reference to the true binding characteristics of the column. The results are shown in Fig. 11 where it is seen that very much more antibody can be bound by the column than was observed in previous experiments. A total of 295 mg of protein was eluted in the A fraction. This gives some indication of the capacity which can ultimately be expected.

Frontal separations. In the discussion of general methods of fractionation it was suggested that a complete frontal analysis of a column be

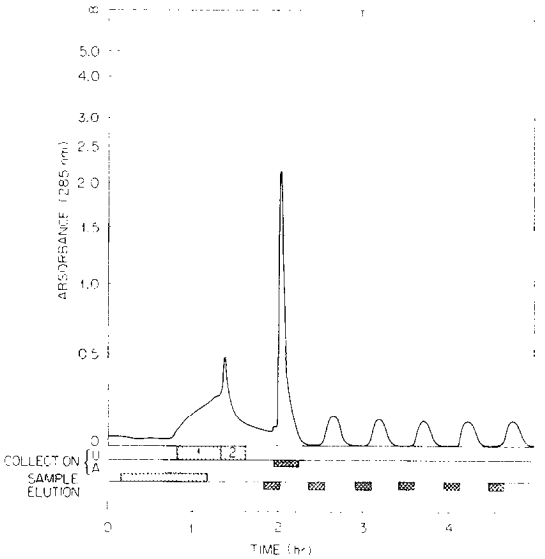


FIG. 12. Unbound protein from experiment shown in Fig. 11 passed through column of immobilized fetal calf serum. Note that protein slowly saturates column and then breaks through as a peak. U fraction collected in two parts as indicated. Thiocyanate (3 M) then used for elution to give large A peak followed by five additional cycles of 3 M thiocyanate to show that column was indeed stripped.

done first and the results used to dictate volumes of sample applied. However, as has been shown, when up to 5 ml of antiserum is applied, all species of antibody with the apparent exception of one were adsorbed on the column. It was of interest therefore to see if these results applied to the repeated cycles described in Fig. 10. The combined antibody (A) peaks from Fig. 10 had been found to contain all species of antibody found in the starting mixture, and had been used to study column capacity (Fig. 11). However the possibility remained that the unbound material shown in Fig. 11 was not active antibody. Therefore this fraction was passed through the column again in unconcentrated form in a total of 105 ml. Should this material have been unreactive, only one long flat peak should have been observed. Instead, the breakthrough of protein was greatly retarded when compared with unbound material of other experiments (Fig. 12), and showed a slow rise to an abrupt peak. On elution with thiocyanate additional material was seen. Two parts of the U peak and the A peak were concentrated and reacted against fetal calf serum by double diffusion. The results (Fig. 13) show that the A peak in Fig. 11 (i.e., the antibodies eluted in the column capacity study) appears to contain all species of antibody present in the starting mixture. The U fraction (unbound in the capacity experiment) when repassed through the column (Fig. 12) contained antibodies to only about four antigens.

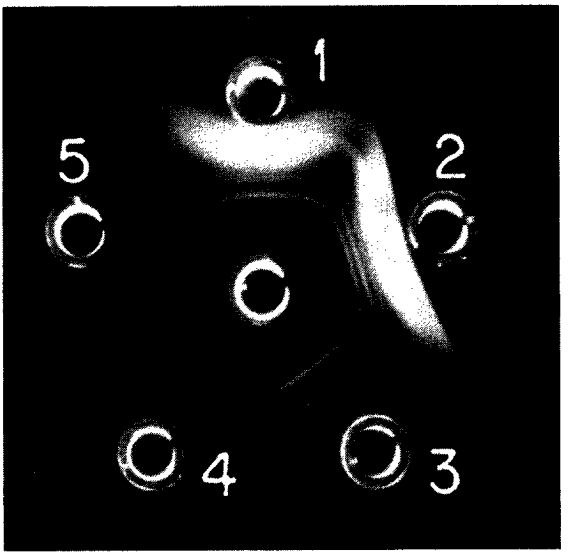


FIG. 13. Ouchterlony analysis of antibodies from separations shown in Figs. 11 and 12. Center well contains fetal calf serum with peripheral wells loaded as follows: 1. Rabbit anti-fetal calf serum from pool of antiserum used for all studies in this paper. 2. Antibodies from A peak of Fig. 11 showing that all antibody types in the original antiserum are recovered from calf serum column. 3. Antibodies in A peak from experiment shown in Fig. 12 showing that three (and possibly a trace of a fourth) antibodies are present. These are antibodies present in excess in experiment of Fig. 11 but which are then shown in Fig. 12 to still be able to bind to FCS column. 4. Fraction U 2 from experiment of Fig. 12. 5. Fraction U 1 from Fig. 12. Results from wells 4 and 5 show that two antibodies appear to be present in almost equal proportion and to overload the column at the same time. However one antibody (compare wells 3 and 4) was removed completely during passage through the column.

The antibodies which were not bound in the experiment in Fig. 12 were divided into two groups—those breaking out first, and those appearing near the end of sample addition. As shown in Fig. 13 those appearing first are two antigens, those appearing later are four. This again demonstrates the power of the combination of frontal separations and cyclic affinity chromatography.

One fraction remained to be studied. The combined U fractions from the multiple-cycle experiment (Fig. 10) were passed through the column without concentration and the results are shown in Fig. 14. The U and A fractions were concentrated and were found on double-diffusion analysis to give two and one lines, respectively (Fig. 15). This demonstrates that a concentration of antiserum can be determined such that all antibodies except one or possibly two will break through and that that volume is slightly less than 5 ml with the serum and column used.

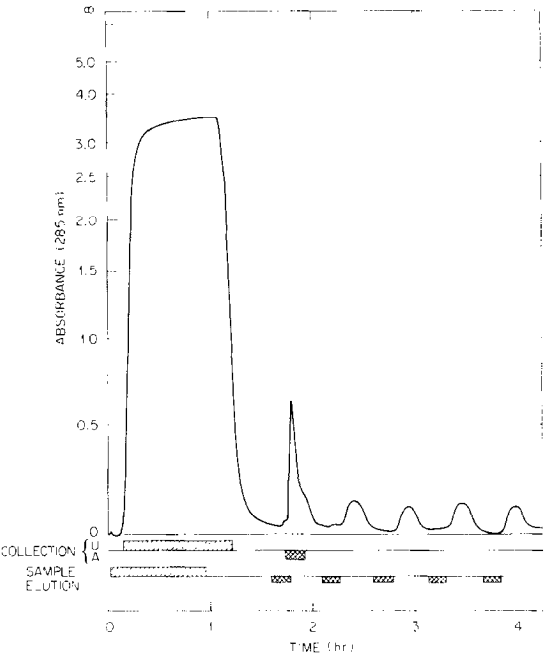


FIG. 14. All U fractions from multiple-cycle experiment shown in Fig. 10 combined and passed in unconcentrated form through FCS (fetal calf serum) column. Note large U peak as expected. Small A peak collected suggests at least one antibody was not completely removed during initial cycles. Fractions concentrated and examined by double diffusion as shown in Fig. 15.

DISCUSSION

A complete system and a range of techniques for the fractionation of mixtures and the purification of antigens and antibodies by cycling affinity chromatography are presented. To explore and demonstrate the basic principles we have used a mixed-antigen column (fetal calf serum covalently linked to Sepharose 4B) and rabbit antiserum against the whole mixture. The automatic Cyclum system allows experimental conditions for adsorption and elution to be more easily studied and for conditions once established to be reproduced precisely. Using 3 M ammonium thiocyanate at pH 7.0, rapid cycling has been found feasible, with efficient recovery of antibody activity and with no appreciable column degradation. The application of this system to a variety of separations including isolation of α-fetoprotein, CEA, and a variety of tissue proteins has been described in preliminary form and will be presented in detail in subsequent papers. Many aspects of this work need careful investigation. These include rates of adsorption and desorption of protein from columns to determine whether intermittent flow, or changes in flow

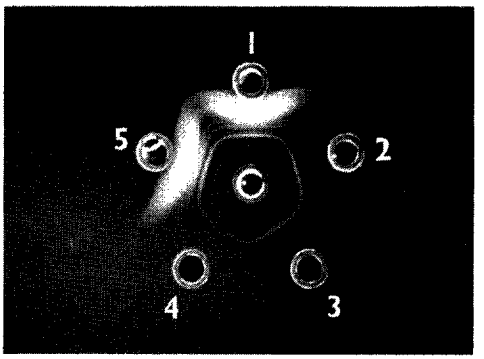


FIG. 15. Demonstration of antibody fractionation using Cyclum and column of fetal calf serum bound to Sepharose. Center well contains whole fetal calf serum. Peripheral wells loaded as follows: 1. Rabbit anti fetal calf serum. 2. Concentrated antibodies from A fraction of Fig. 14. One major and one very minor band observed. 3. Concentrated U fraction from Fig. 14 experiment showing that antibodies against one antigen are present in excess and that monospecific antisera can be obtained by exploiting natural unbalance of antisera. 4. Concentrated A fraction obtained by passing all U fractions from experiment shown in Fig. 6. Line of identity with well 3 shows same antibody again isolated. 5. Antibodies in A fractions from Fig. 6 bound to FCS (fetal calf serum) a second time, eluted and concentrated to show, by comparison with well 1, that all species of antibody may be bound twice without losing any antibody species and also that FCS column, after prolonged use, still binds all species of antibody.

rate during a cycle may make faster cycling possible, and for elution of peaks in smaller volumes.

The difficulty of high-affinity antibodies which gradually block an antigen column has not been encountered in practice.

The stability of columns thus far studied has been surprising. In one instance an immobilized antibody column was left for 24 hr in 1.5 M thiocyanate by accident with no loss in capacity. Since prolonged exposure of eluted antibodies to thiocyanate has proven deleterious, the question of why immobilized antibodies and antigens are so stable requires some comment. Eluted proteins are probably at least partially unfolded when in thiocyanate, and in this condition would interact with each other except for the presence of this chaotropic ion. If the concentration of this latter can be very rapidly reduced, as occurs in fiber dialyzers, some critical concentration may be passed before much interaction occurs. On the column, conditions are quite different. The immobilized protein, even if completely uncoiled, would have no protein in solution to interact with as the thiocyanate concentration is reduced. Presumably it refolds without interaction with nonimmobilized protein and regains its native configuration.

These studies define usable conditions which can serve as a starting point for studies aimed at the optimization of fractionation and prepara-

tive work based on the methods presented. We have used fetal calf serum proteins here because it is our intention to develop high-capacity systems for removing fetal calf serum proteins from tissue-culture fluids as part of a study of tumor-associated antigens released into the medium by tumor cells.

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