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TECHNIQUES AND INSTRUMENTATION FOR PREPARATIVE IMMUNOSORBENT SEPARATIONS

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CONTENTS

1. INTRODUCTION

In celebrating the 500th Anniversary of the University of Uppsala, it is appropriate to recognise that the progress I would like to present today is a direct consequence of a serendipitous discovery made within its walls by our distinguished host, Dr. Porath, a Mr. Sundberg and the chairman of this session, Dr. Kristiansen. Acting on a suggestion of Dr. Rolf Axen, they treated polymeric carbohydrates with cyanogen bromide under alkaline conditions and discovered what was to become the most widely used and successful means of preparing immobilised reagents for affinity chromatographic separations[1].

Since its discovery ten years ago, the cyanogen bromide activation of agarose has provided a ready means for the convenient synthesis of immunosorbents destined to be utilised in preparative separations of macromolecules or for associated analytical purposes. The provision of a consistently reliable spherical preparation of agarose, namely Sepharose manufactured by Pharmacia, has ensured an increasing utility of the method in comparison with alternative supports and other chemical strategies. Seven years ago our laboratory began utilising immunosorbents for the specific isolation of immunological reagents relevant to the diagnosis and understanding of human neoplastic disease[2]. The simplicity and preparative advantages offered by these biospecific methods prompted an associated research programme into the chemical and physical characteristics of the immunosorbents and in the methodology of their efficient use in preparative separation protocols. As the demand for separations and purifications increased, associated automatic instrumentation was developed to enable unattended routine processing of samples and antisera.

It is the purpose of this contribution to firstly report on some of the more significant findings in the technique of efficiently applying immunosorbents to the preparative separation of pure antigens and monospecific antibodies and subsequently to describe the evolution of the instrumentation designed for this purpose, culminating in a soft-programmable repetitive chromatograph of wide versatility, which is being

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introduced here publicly for the first time. In conclusion, an attempt will be made to assess two future applications of immunosorbents in improving diagnostic methods and their potential in the therapy of human disease.

The data to be presented on immunosorbent characteristics has been previously published in an internal report (ORNL-TM-4962) and is treated more fully in ref. 3.

2. CHEMICAL AND PHYSICAL CHARACTERISTICS OF SEPHAROSE BASED IMMUNOSORBENTS

Although we have investigated the potential of a wide range of support materials and activation chemistries for preparing immunosorbents, we have found that three types of support material satisfy our preparative requirements adequately. Most generally applicable are immobilised reagents prepared from cyanogen bromide activated Sepharose 4B. Where a high degree of rigidity is required for specific applications, controlled pore glass is preferable and similarly, when rapid reaction rates are desirable, solid glass spheres offer definite advantages over the porous supports. Despite many attempts to improve on the original methods of activating Sepharose[4,5], we have been generally unsuccessful and now routinely use the alkaline buffer technique of Porath *et al.*[6]. Considering the extra chemistry involved, the use of molecular extenders is hardly justified for preparative immunosorbents by the small increase in reactivity observed, particularly in comparison with other factors which will now be discussed.

In the application of antibody immunosorbents for preparative purposes, the specific reactivity of the immobilised species is an important operating parameter. Any reduction in reactivity will be reflected in a lower capacity for antigen and hence a diminished overall yield in addition to be wasteful of what may well be a valuable reagent. We have found that the specific reactivity of immobilised antibodies is inversely related to the protein density on the support. Thus, the greater the amount of antibody linked to a given volume of support, the less its specific immunological reactivity to antigen becomes. This effect is shown in Fig. 1 in which the specific reactivity, expressed as a molar ratio, of an immobilised IgG fraction of a high-titre goat anti-human albumin sera is shown as a function of increasing ligand loading.

It is apparent from this result and from similar experiments using other immunological systems that the effect is a general one and occurs on most porous supports. Using monospecific antibody preparations, the maximal reactivities achieved at low antibody densities for protein antigens are in the region of 1.20 expressed as a molar ratio. This 60% theoretical activity is reduced to less than 25% at the maximum loading on both Sepharose and porous glass; on solid matrices the effect is less pronounced, unless of course a monolayer of reagent is exceeded. A similar effect is observable with antigen-loaded immunosorbents and is probably due, in both cases, to steric hindrances accentuated by the tortuous nature of the pores within the support.

The conditions used in preparing the immunosorbents have some bearing on the loss in reactivity. The data used in Fig. 1 were obtained by limiting the degree of activation of the Sepharose by restricting the amount of cyanogen bromide used. If a constant high degree of activation is employed and the available protein restricted during the subsequent coupling reaction, there is less variation in reactivity although

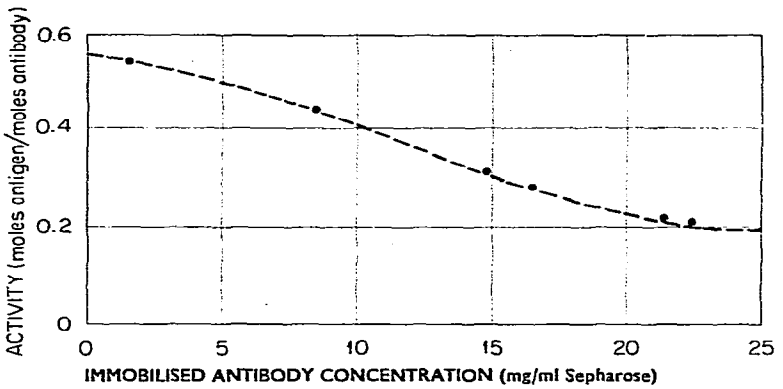

Fig. 1. Effect of increasing substitution of antibody on its immunochemical activity.

the overall values are somewhat lower in comparison with adoption of the previous strategy. These findings are of practical significance where a limited supply of reagent to be immobilised is available and maximum preparative efficiency is demanded.

Of more fundamental importance to the separational efficiency of immunosorbents is the inherent affinity constant spectrum of the antibody preparation being used. Although these equilibrium constants are many orders of magnitude greater than their equivalent counterparts in partition and ion-exchange chromatographic techniques —making the term chromatography when using immunosorbents somewhat debatable— they nevertheless dictate an equilibrium process to be considered in using immunosorbents. Their values become of significance in practical terms when very low concentrations of soluble component exist as exemplified in immunosubtractive procedures in which the removal of a trace antigen is required. In conventional preparative applications, however, the numerical value of the constants is only relevant in predicting purity of product and assessing the efficiency of eluting solutions in dissociating the immobilised complex. For these purposes it is not necessary to obtain a highly precise value, defined in chemical kinetic units, as relative values determined under similar experimental conditions are acceptable and pragmatically adequate.

Fig. 2 demonstrates the practical use of such information. This composite plot of bound antigen, expressed in convenient mass per unit volume units *versus* free antigen concentration, was constructed from values of affinity constants measured in various buffers including potential eluents. From Fig. 2, it is readily apparent that the chaotropic solutions of thiocyanate ion are considerably more effective than urea in displacing bound antigen at all free antigen concentrations. The value of this type of direct presentation, as opposed to Scatchard and other immunochemical interpretations, is that it allows ready prediction of efficiencies and thereby ultimate yields in practical terms and units. The intrinsic affinity constant of a particular antibody is of course dependent largely upon the idiosyncratic response of the immunised animal, and thus is beyond our control. However, later in this contribution the potential of antibody fractionation dependent upon affinity constants will be discussed, thus offering an alternative to this present constraint.

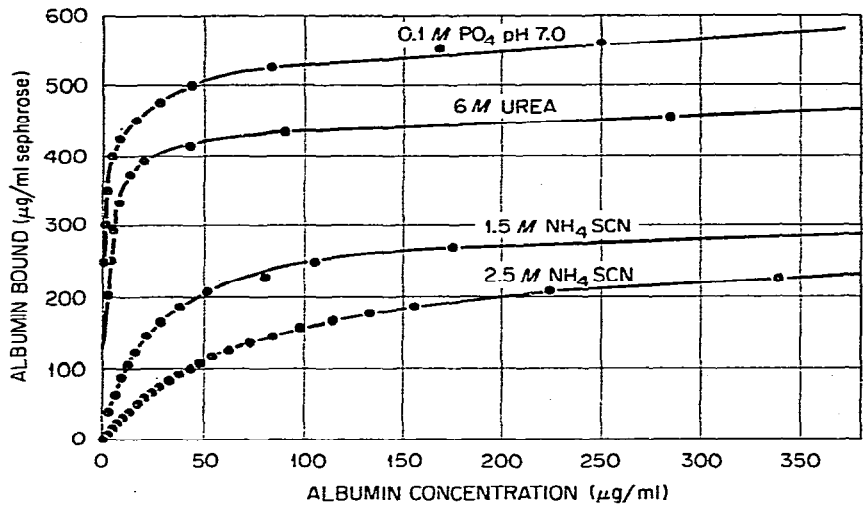

Fig. 2. "Direct plot" of albumin bound to specific immunosorbent expressed in weight per volume units as derived from affinity constants.

The largest restriction imposed by the use of immunosorbents based on porous supports is a function of the low diffusibility of macromolecules in solution. This effect is illustrated in Fig. 3 where the overall reaction kinetics of albumin with an anti-albumin Sepharose 4B immunosorbent is depicted. It is apparent that the overall reaction can be considered as biphasic; the first 80% of the reaction occurring at a faster rate than the final 20%. The finding that chaotropic dissociation, also depicted in Fig. 3, mimics the adsorption profile indicates diffusion rather than immunochemical reaction as the rate limiting parameter. This finding has significant implications in applying porous immunosorbents to preparative separations by imposing a time restriction on the process. Solid support-based immunosorbents show a much faster reaction, limited only by interstitial diffusion, but their much reduced capacity tends to negate this potential advantage.

The diffusion dependence makes its contribution felt in practical terms by lengthening the adsorption time for maximum reaction and extending wash protocols to remove contaminants. The strategy that we have evolved in overcoming these restraints is to attempt to limit reaction to the outer regions of the immunosorbent bead and thus take advantage of the more rapid reaction rate. This is most readily achieved in column operation by an increase in flow-rate and thereby limiting the exposure of sample to the surface of the immunosorbent beads and concurrently restricting contaminant diffusion into the deeper pores. The loss in effective column capacity resulting from this strategy is amply compensated by the increase in overall yield that is obtainable.

Using the automatic repetitive chromatographic systems to be described shortly, adoption of this high flow-rate procedure has resulted in a four-fold increase in yield over the earlier standard protocols. Using a 100-ml column, it has become routine practice to isolate gram quantities of monospecific antibodies of better than 95% purity on a daily basis. The quality of these products can be improved further

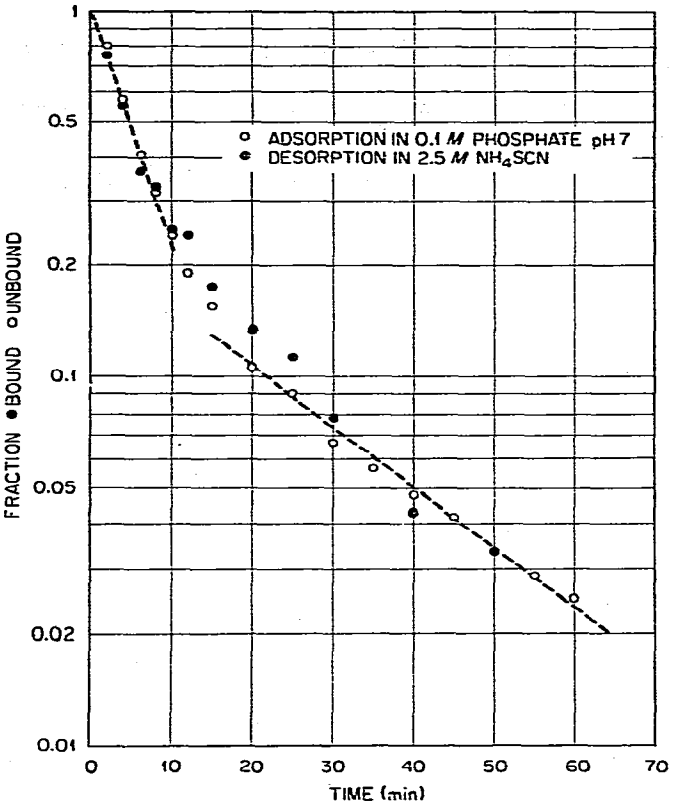

Fig. 3. Adsorption and desorption of albumin from its specific immunosorbent under stirred-batch conditions.

by incorporation of non-ionic detergents in the buffers, to reduce non-specific adsorption, and by removal of minor contaminants by reprocessing or by conventional protein chromatographic procedures.

Before describing the chromatographic systems and the associated instrumentation, there is a chemical strategy that I would like to share with you that can reduce the time and processing effort in preparing immunosorbents. This involves the reversal of an antigen immunosorbent directly into a specific antibody immunosorbent by the simple means of chemical crosslinking. The immobilised antigen is reacted with an antisera containing the specific antibodies and after washing to remove contaminating proteins is treated with a dilute solution of a divalent cross-linking agent such as glutaraldehyde or dimethyl suberimidate, the process being performed either as a batch operation or directly in a column. After a further wash with chaotrope and buffer, the unreacted groups of the cross-linking agent are blocked with an amine or hydroxylamine in the case of glutaraldehyde. The original antigen immunosorbent can now be used as specific antibody immunosorbent. Table 1 shows typical results of this reversal process as applied to albumin immobilised on Sepharose 4B; details of the procedure will be published elsewhere[7]. As one of the immunodeterminant

TABLE 1

EFFECT OF CROSS-LINKING AGENT CONCENTRATION AND REACTION TEMPERATURE ON IMMUNOSORBENT REVERSAL

1-ml samples of HSA-Sepharose were reacted for 1 h with 1.0 mg of purified goat anti-HSA antibodies and cross-linked with glutaraldehyde at pH 8.8 for 1 h.

Temperature (°C)	*Cross-linking agent conc. (M)*	*IgG cross-linked (μg)*	*Cross-linking efficiency (%)*	*Reactivity (moles/mole)*
4	0.001	380	75	0.39
4	0.005	415	80	0.51
4	0.01	445	87	0.46
4	0.02	450	85	0.46
4	0.05	415	79	0.45
25	0.001	375	83	0.52
25	0.005	380	88	0.46
25	0.01	440	88	0.46
25	0.02	455	89	0.47
25	0.05	385	85	0.47

sites of the antibody is involved in the cross-linking to antigen, the resultant reactivity is halved as can be seen in Table 2, which compares the specific reactivities obtained using this procedure with those resulting from the more conventional direct elution and immobilisation process. It is to be noted that the reactivity dependence on protein density is maintained in this reversal strategy. This simple procedure has proved consistently reliable and is to be recommended as an expedient method where the loss of immunosorbent capacity can be tolerated by the savings of time and effort involved in conventional elution and re-immobilisation.

TABLE 2

COMPARISON OF THE REVERSAL METHOD WITH THE DIRECT METHOD

Averages of 1-ml samples of Sepharose prepared at 25°.

Direct		*Reversed*	
IgG (mg)	*Reactivity (moles/mole)*	*IgG (mg)*	*Reactivity (moles/mole)*
0.9	1.10	1.0	0.57
1.8	0.70	2.0	0.35
3.5	0.60	3.7	0.34
6.8	0.54	7.0	0.29
12.3	0.50	11.7	0.24

3. AUTOMATIC PREPARATIVE INSTRUMENTATION

The requirements of an instrument system dedicated to preparative separations utilising immunosorbents are comparatively simple. They include a means of sequentially applying sample, washing buffer and eluting buffer to the immunosorbent

column, facilities to separately collect the unadsorbed eluent and the desorbed product and provisions for monitoring and controlling these operations. Schematically the system can be presented as shown in Fig. 4. As the process invariably involves repetitive operation, due to the limited capacity of the immunosorbent column, additional means for predetermining the number of cycles to be performed and continuous dialysis of the product are conventionally incorporated into the design.

In using the high flow-rate rapid cycling strategy, referred to earlier, the volume of eluted fractions can be considerable. A modification of the original system

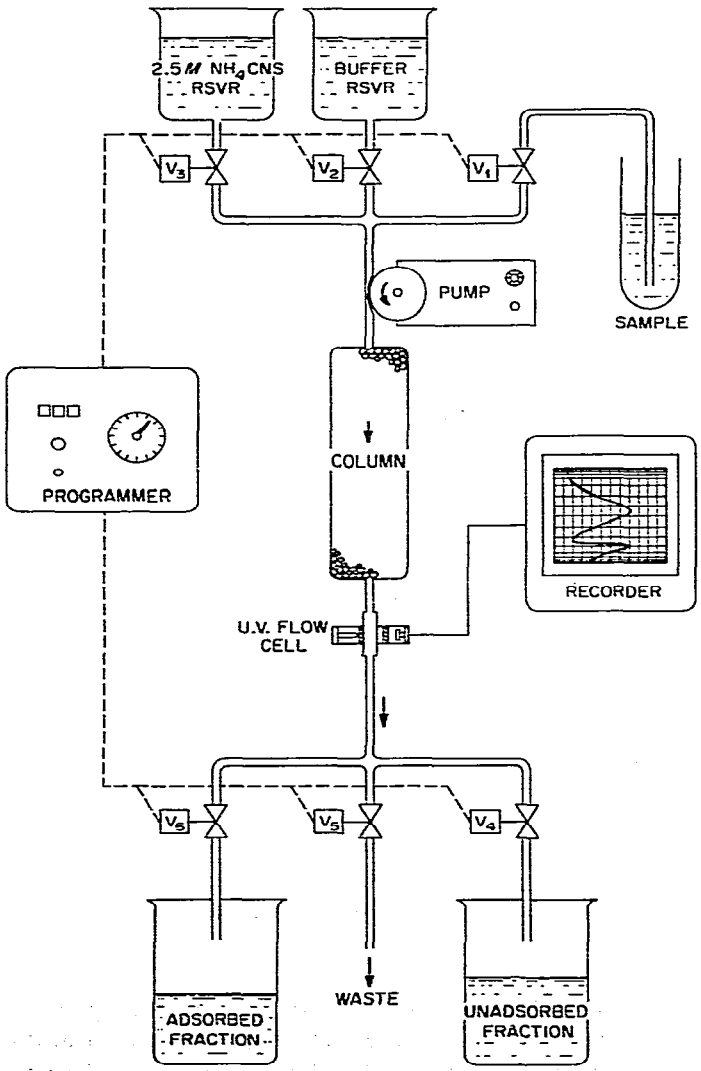

Fig. 4. Schematic representation of preparative affinity chromatographic system.

in which a hollow-fibre cartridge is incorporated into the design, as shown in Fig. 5, largely overcomes this problem. A back-pressure is created in the cartridge by valving the dissociated fraction to a second channel of the peristaltic pump that has a lower flow-rate than the primary column delivery. The resultant internal pressure within the hollow fibres provides continuous dialysis and, more importantly, concentration of the fraction. In practice, a concentration factor of about five is possible without damage to the integrity of the fibres.

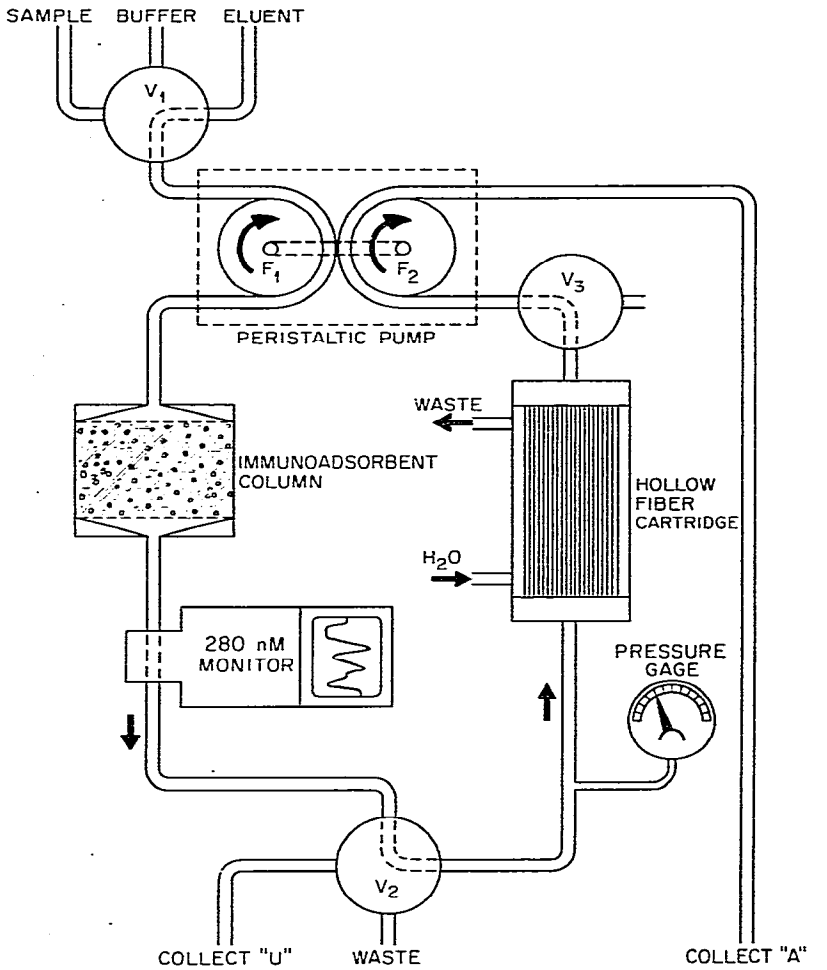

Fig. 5. Schematic diagram of continuous dialysis and concentration preparative system.

The earlier instrument systems, which are described in detail by Anderson *et al.*[8,9], were constructed using cam-activated programmers in conjunction with automatically resetting clocks. An example of one of these earlier models is shown in Fig. 6. Although adequate for long-term routine processing of antigen preparations

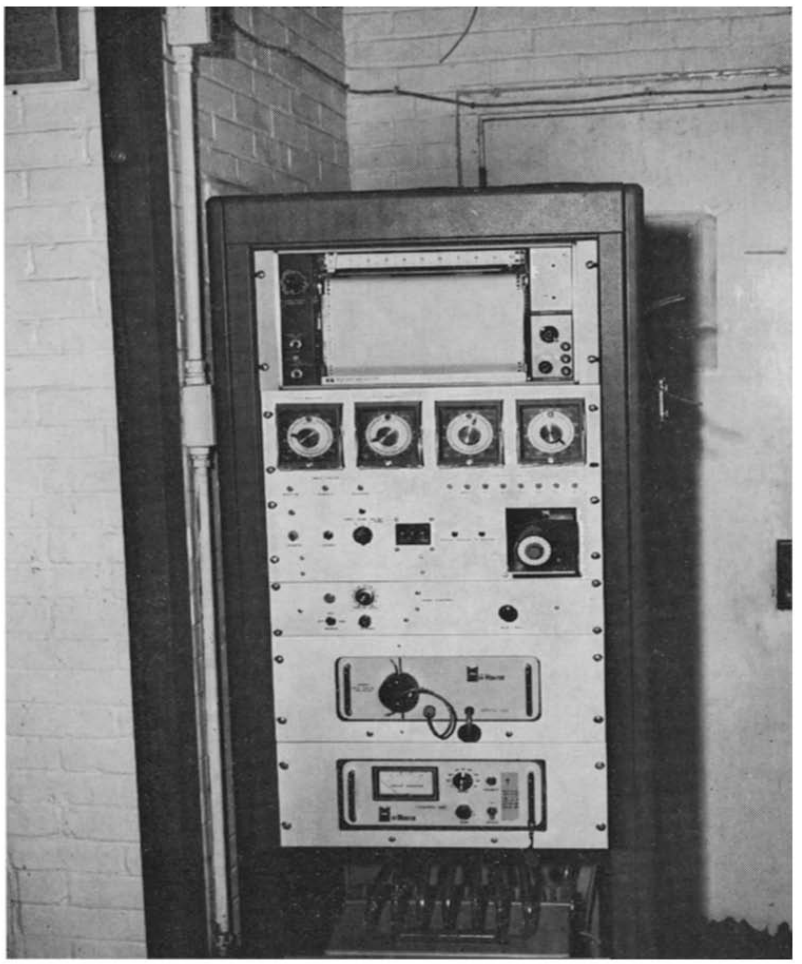

Fig. 6. Early automatic immuno-affinity system.

or antisera, they suffered from the disadvantage of cumbersome programming and restrictions in flexibility for small-scale research applications.

The next generation of automatic immuno-affinity instruments, by now known as "Cyclums", incorporated a more easily adjustable programmer and an increased versatility in operational parameters. A circular-faced timing unit not only provided a visual representation of the total programme but further provided facilities for overlapping functions and for minor timing adjustment during the operation of the system. These latter improvements are essential in optimisation of the protocols in the initial setting-up operation. Several free-standing systems, as illustrated in Fig. 7, were constructed and are in use in a few laboratories in the U.S.A. under collaborative research programmes. A smaller bench-top system was made for the application of low-volume columns, where a higher degree of resolution was necessitated;

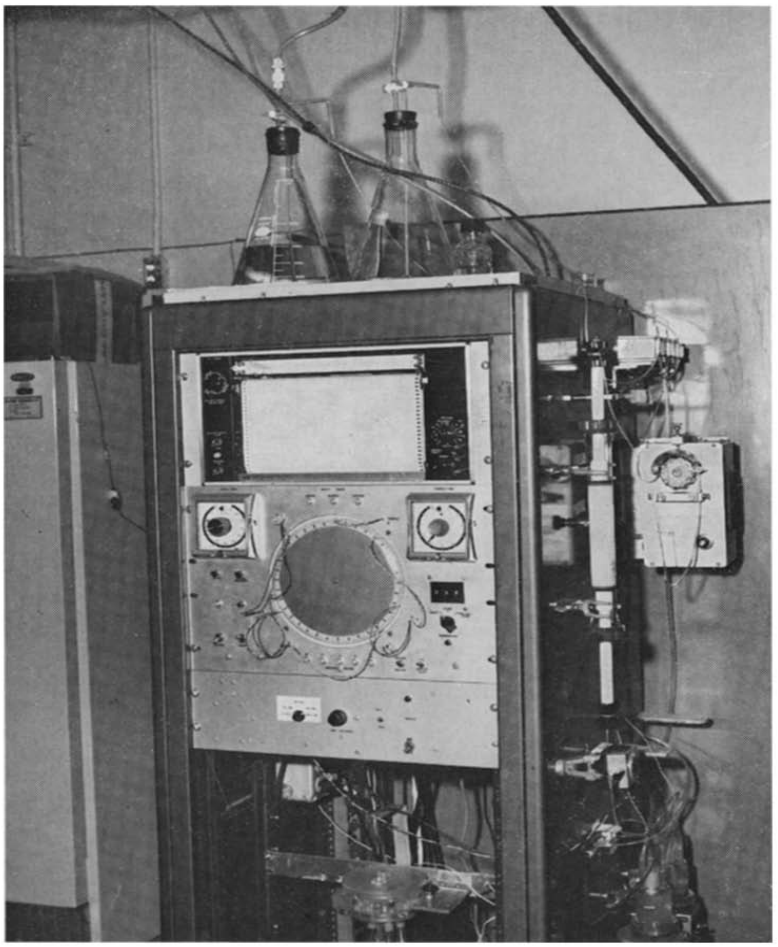

Fig. 7. "Cyclum" automatic immuno-affinity chromatography system with rotary programmer.

the single model constructed is shown in Fig. 8. Finally, in this generation of instruments, a solid-state digital programmer was developed that had the advantage of no mechanically moving components and high reliability in operation. All of these instruments were however dedicated systems, their application being restricted to conventional repetitive immunosorbent separation procedures.

Our increasing awareness and technical experience in exploiting immunosorbents for preparative applications prompted the development of a new concept in chromatographic separation technology, that I now would like to present briefly for the first time in public. This instrumental system, which we have christened the ARK (Automated Repetitive Chromatograph), extends the principle of the immuno-affinity instrument systems into all areas of preparative chromatography.

Basically we have designed an instrument system that can be pre-programmed in "users language" to perform most, if not all, of the procedures required in any

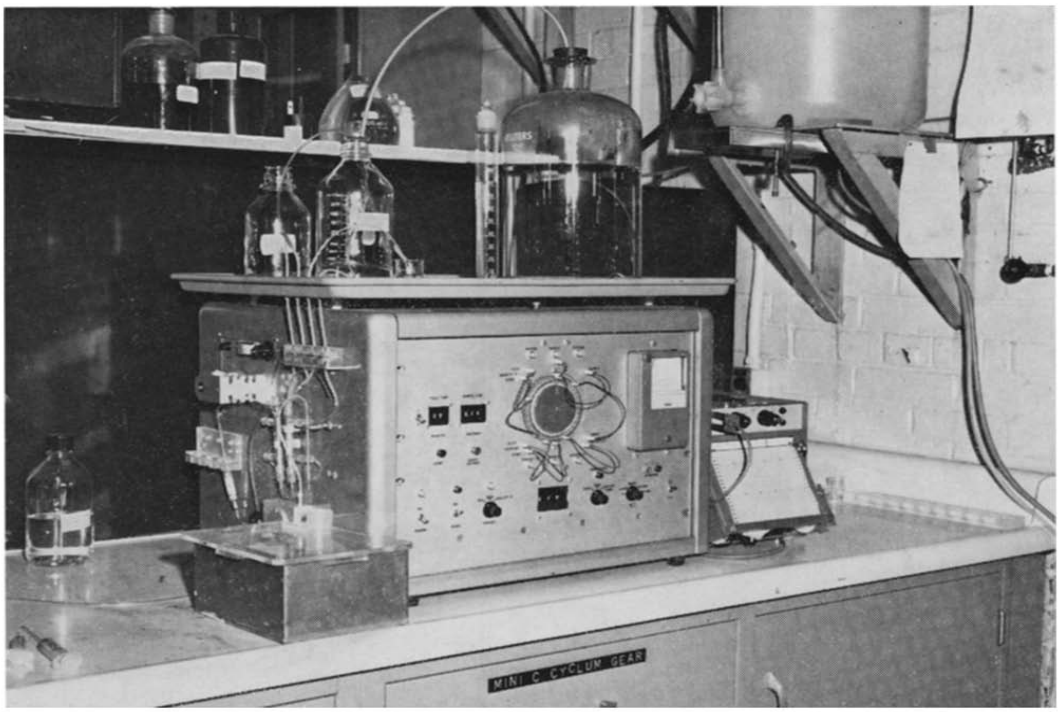

Fig. 8. Bench-top miniaturised automatic immuno-affinity chromatography system.

chromatographic separation in which the separation media can be considered reusable or regenerable. To obviate the dependence of column dimensions and capacity on sample size or concentration, we have adopted the principle of using a standard column and repeating the separation until the total sample has been processed.

Using this standard column, the operating protocol can be optimally predetermined for the required separation and stored as a "machine language" programme for later reactivation and practical use. The encoded programme contains all essential operating and system parameters, such as fluidic connections, timing intervals, collection requirements and general housekeeping duties of the instrument. Safety features, such as fluid detectors and synchronisation signals, ensure reliable and unattended automatic operation with preservation of valuable samples and products under unforeseen circumstances. The only variable remaining to the investigator in reactivating a separation, is to adjust the sample concentration, or the application volume, to a value within predetermined optimal limits.

The basic design of an instrument to give this operational versatility can be conveniently divided into two elements, the hardware and the software facilities. In using these terms, we intentionally are plagiarising the language usually associated with computer design and operation, the analogy with this technology being convenient as well as appropriate. In principle, the hardware of the ARK is fairly conventional and is schematically shown in Fig. 9. The input and output versatility of

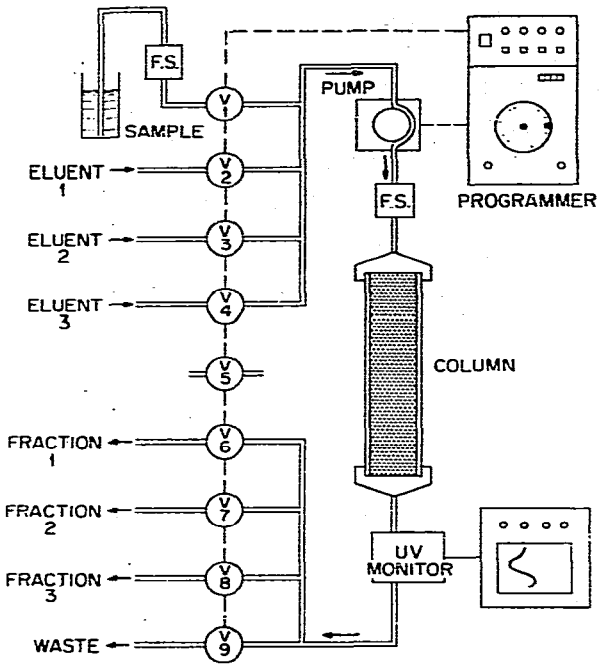

Fig. 9. Schematic representation of Automatic Repetitive Chromatography (ARK) system.

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   ARK PROGRAM:   SIMPLE IMMUNOSORBENT SEPARATION

LINE    TIME                  INSTRUCTION
0010     T1     IN: SAMPLE;   OUT: WASTE;
0020     T2     IN: BUFFER;   OUT: WASTE;
0030     T3     IN: BUFFER;   OUT: COLLECT U;
0040     T4     IN: SCN;      OUT: COLLECT U;
0050     T5     IN: SCN;      OUT: DIALYZE A, COLLECT A;
0060     T6     IN: BUFFER;   OUT: DIALYZE A, COLLECT A;
0070     T7     IN: BUFFER;   OUT: WASTE;
0080     T8     IF: FS1 = 1, RESET TO 0010; ELSE: STOP;
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Fig. 10. User language program for simple immunosorbent repetitive separation.

the column is determined by an array of fluid switches under the control of the software programmer. Fluidic sensors (F.S.), consisting of small encapsulated light sources and detectors that signal the presence of air in fluid lines, are strategically placed in the circuit. These devices continuously monitor significant fluid lines and inform the programmer when the sample has been completely processed. Conventional UV monitoring facilities and a multi-channel peristaltic pump complete the basic instrumental system.

The relationship between the hardware components of the system and the software programming is best described by using a specific example of a separation. The user language programme for a simple immunosorbent repetitive separation,

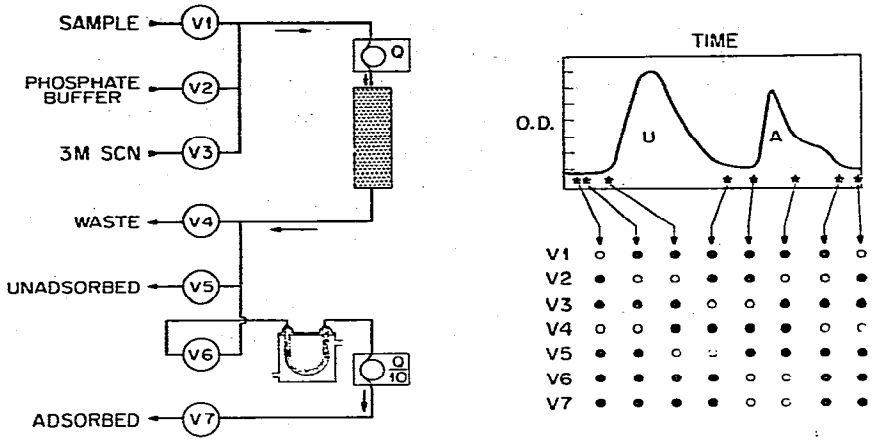

Fig. 11. Fluidic circuit and program logic for simple immunosorbent separation on ARK.

with dialysis and concentration of the desorbed product, is shown in Fig. 10. Here the logic of the required functions is defined in terms directly relevant to the separation. The timing sequence (T1–T8) is to be determined empirically and is dependent upon the capacity of the immunosorbent, the column flow-rate, and the degree of purity required in the product. Once these times have been determined, however, they become an integral part of the software programme and are thus retained for future re-use of the column.

In translating these requirements into an operational programme, a fluid circuit diagram and a machine language listing is required. This is illustrated in Fig. 11 for the simple immunosorbent separation including dialysis and concentration. The inputs to the column and the collection requirements are now defined as practical

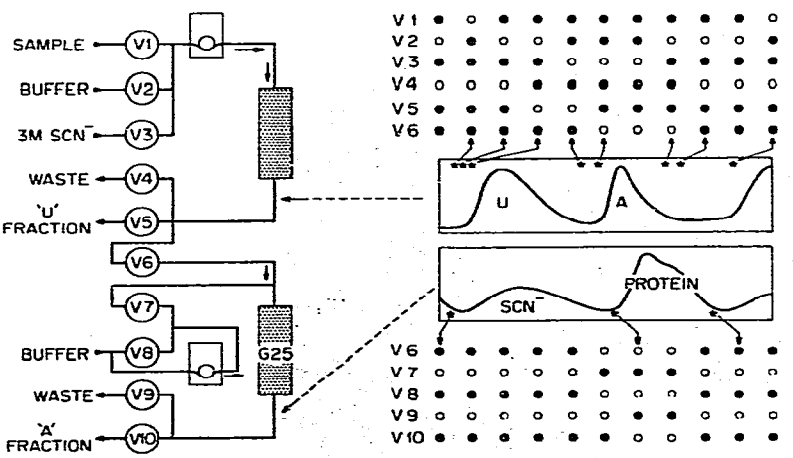

Fig. 12. Fluidic circuit and program logic of immunosorbent separation with concurrent gel filtration of desorbed product.

connections to the array of fluid switches. The sequential operation of this array is reduced to a matrix of switching instructions to be activated at the time intervals stored in the programmer. This matrix is shown at the right of the figure related to the output of the column; the input instructions are of course anticipatory and preceed the output signal by a constant interval dependent upon the void volume of the column. The programmer controls not only the fluid switch array but is further programmed, by means of subroutines, to respond to instructions that encompass safety logic, recycling conditions and other specific requirements.

One of the major advantages of the system is the capability of programming two or more separation columns in serial operation. This is illustrated in Fig. 12 which shows the circuit and logic for the immunosorbent separation with concurrent

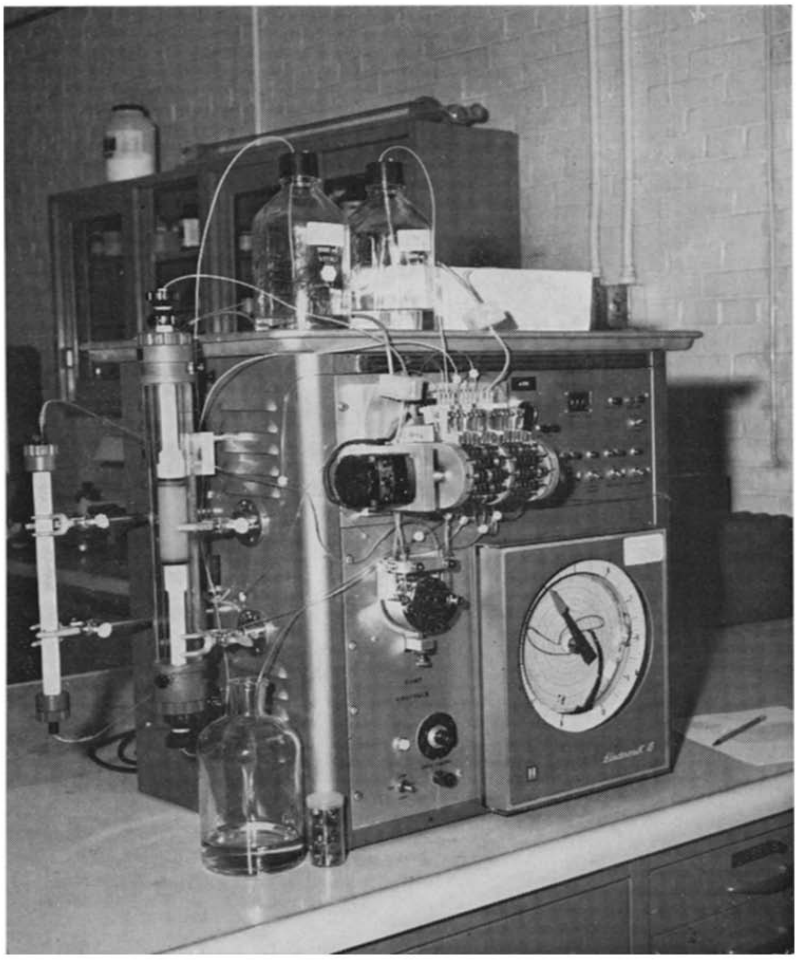

Fig. 13. Prototype Automatic Repetitive Chromatograph.

gel filtration of the desorbed product to remove chaotrope ions. Although the fluid circuit and the operational programme appear complex, in practice it is only marginally more difficult to optimise operating conditions compared with a single column separation. In this context, it is advantageous to tailor the secondary column to suit the elution characteristics of the primary separation and thus retain efficient synchronisation and overlap of functions.

The prototype instrument that we have constructed employing these concepts is shown in Fig. 13. The repetitive separation being performed is that of an immunosorbent column in conjunction with a Sephadex G25 gel filtration, as detailed in Fig. 12. The two columns can be seen on the left, and a multi-way programmable valve and a circular chart recorder on the front of the instrument. Above the circular recorder, which is additionally adapted to be a time programmer, is the main control panel and on its left the peristaltic pump. The output of the immunosorbent column is connected through the minaturised UV monitor, the signal of which is being recorded on the circular chart recorder. The variable rotation time of this chart is presettable and, by means of a peripherally placed sequence of holes, photometrically provide the required timing signals.

The multi-way programmable fluidic valve that we have used in this prototype, is shown in more detail in Fig. 14. By means of lever mechanisms, fluid lines

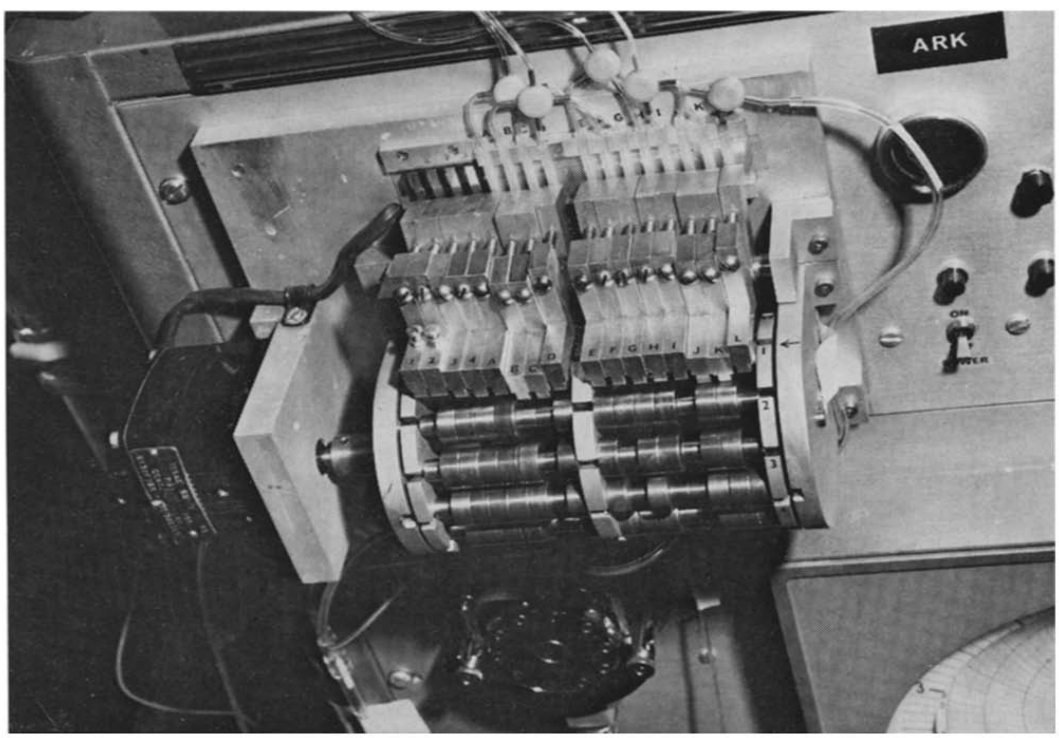

Fig. 14. Multi-way programmable fluidic valve.

are opened or closed by prior insertion of small- or large-diameter rollers on the drum shafts. The order of these rollers along a particular shaft is dictated by the software program discussed earlier, the shafts corresponding to each operational sequence. Upon receipt of a timing signal from the programmer, the drum indexes one position thus opening or closing the fluid lines according to the pre-programmed sequence. This valve has twelve fluid channels with a further four micro-switched electrical channels and a repetitive indexing sequence of twelve operating modes. We have found this degree of versatility to be adequate for all of the chromatographic separations that we have attempted. It is obvious that there are several alternative means of improving the hardware components of this prototype and we are currently investigating the application of microprocessor technology to the design of a fully automated and readily programmable instrument.

My colleague, Mr. David E. Levy, has successfully programmed the following preparative separations on this prototype in addition to the immunosorbent protocols previously outlined. Gel filtration for both simple desalting and more complex fractionation of protein solutions; separation of IgG on a routine basis using QAE-Sephadex and ethylenediamine buffers for elution with acetate buffer for regeneration; isolation, with concurrent concentration in a secondary column of specific proteins using DEAE-agarose and a step-wise buffer elution sequence; fractionation of ribonucleic acids on a hydroxyapatite column using a regenerable gradient subroutine. In addition to these established separations, application of the instrument to specific requirements in immunosubtraction and depletion studies have further justified the development of the system.

In conclusion, I would like to introduce briefly two future applications of immunosorbents that we are now studying. The idiosyncratic response of immunised animals is a constant source of irritation to an immunochemist dependent upon this biological system for his reagents. To obviate this dependence, we have initiated a programme with the objective of fractionating the spectrum of antibodies produced in immunisation into populations of defined affinity and, to a certain extent, specificity. Theoretical explorations have outlined the parameters necessary for this separation and we are currently designing a practical system based on these feasibility studies. Briefly it appears that a continuous counter-current system using antigen immunosorbents will provide the required preparative capability, although a true chromatographic approach has not been entirely ruled out. The justification for this effort is related to the improved sensitivity of radioimmunoassays promised by the defined high-affinity fractions that result from the separation.

The other future application of immunosorbents is related to *ex vivo* immunodepletion of specific antibodies from immunised animals and to a potential therapeutic strategy for the amelioration of certain human diseases. These applications are made possible by a blood cell separator program within our laboratory. The major objective of this latter program has been to improve granulocyte separation capability for replacement therapy of cancer patients undergoing chemotherapy. The blood cell separator however, also provides a continuous cell-free plasma stream into which a sterile immunosorbent containing cartridge can be incorporated for continuous specific immunodepletion purposes. In therapeutic applications the cartridge would remove from the circulating plasma stream specific immune blocking factors such as antigens, immune complexes and idiotypic antibodies that are implicated in the

etiology of neoplasia. Removal of these blocking factors will allow the natural immune defence system to again become effective.

In addition to the human blood cell separator, we have developed a low-volume rotor for the continuous separation of plasma from small animals. Incorporation of an antigen immunosorbent cartridge in this *ex vivo* loop will allow continuous removal of specific antibodies from the animal and thus provide a theoretical increase of an order of magnitude in yield over weekly bleedings. The use of the ARK in this future application of immunosorbents will allow a continuous recycling of two cartridges, thus reducing the total amount of antigen immunosorbent required in the strategy. More important than this potential of an increased yield of antibody is perhaps the biological question as to the immunological response of the animal to this specific depletion with regard to affinity and specificity of the regenerated antibody population.

Seeking answers to questions such as this provides the stimulus in our continuing endeavours to exploit fully the potential application of immunosorbents. An innovative approach, based originally upon a touch of Swedish serendipity, will hopefully provide the answer and hence the reward. It is in this context that the invitation to attend this Symposium and its opportunity to share with you a few of the answers that we have already found, is, in itself, an honour and an ample reward.

4. SUMMARY

Studies of the physical and chemical characteristics of immunosorbents have allowed operational improvements to be made in their application to preparative separation of pure antigens and monospecific antibodies. The loss of immunochemical reactivity with increasing protein substitution and the limitations imposed by the porous nature of many supports, have been significantly overcome by improved techniques and operational protocols. The development and operation of dedicated immuno-affinity chromatographic systems designed for routine preparative applications are described. A new automatic repetitive chromatography system, generally applicable to a wide spectrum of routine biochemical preparative separations, is presented for the first time.

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