Practical Immunology

Frank C. Hay
PhD
Professor of Immunology
St. George's Hospital Medical School
Cranmer Terrace
London

Olwyn M.R. Westwood
PhD
Senior Lecturer in Immunology
University of Surrey Roehampton
and St. George's Hospital Medical School
London

with the assistance of
Paul N. Nelson
Wolverhampton University, Wolverhampton

FOURTH EDITION

Blackwell
Science

Practical Immunology

FIRST EDITION
Sarah-Louise and Rebecca

SECOND EDITION
Alexander and Thomas

THIRD EDITION
Elaine and Frances

FOURTH EDITION
Frances and John

Practical Immunology

Frank C. Hay
PhD
Professor of Immunology
St. George's Hospital Medical School
Cranmer Terrace
London

Olwyn M.R. Westwood
PhD
Senior Lecturer in Immunology
University of Surrey Roehampton
and St. George's Hospital Medical School
London

with the assistance of
Paul N. Nelson
Wolverhampton University, Wolverhampton

FOURTH EDITION

Blackwell
Science

a Blackwell Publishing Company
Editorial Offices:
Osney Mead, Oxford OX2 0EL, UK
Tel: +44 (0)1865 206206
Blackwell Science, Inc., 350 Main Street, Malden, MA 02148-5018, USA
Tel: +1 781 388 8250
Blackwell Science Asia Pty, 54 University Street, Carlton, Victoria 3053, Australia
Tel: +61 (0)3 9347 0300
Blackwell Wissenschafts Verlag, Kurfürstendamm 57, 10707 Berlin, Germany
Tel: +49 (0)30 32 79 060

First published 1976
Second edition 1980
Third edition 1989
Reprinted 1991
Fourth edition 2002

Library of Congress Cataloging-in-Publication Data
Hay, Frank C.
Practical immunology / Frank C. Hay, Olwyn M.R. Westwood;
with the assistance of Paul N. Nelson. — 4th ed.
p.; cm.
Rev. ed. of: Practical immunology / Leslie Hudson, Frank C. Hay. 3rd ed. 1989.
Includes bibliographical references and index.
ISBN 0-86542-961-8 (pbk.)
1. Immunology—Laboratory manuals. I. Westwood, Olwyn M.R.
II. Nelson, Paul N. III. Hudson, Leslie. Practical immunology. IV. Title.
[DNLM: 1. Immunologic Techniques—Laboratory Manuals. 2. Allergy and Immunology—Laboratory Manuals. QW 525 H412p 2001]
QR183 .H39 2001
616.07'9—dc21
2001035417

ISBN 0-86542-961-8

A catalogue record for this title is available from the British Library

Set in 8½/13¼ Stone Serif by Graphicaft Limited, Hong Kong
Printed and bound in Great Britain by MPG Books Ltd, Bodmin, Cornwall

For further information on Blackwell Science, visit our website:
www.blackwell-science.com

CONTENTS

FOREWORD TO THE FIRST EDITION

Immunology might well claim to be the most popular and the most glamorous of biological sciences today. I suspect that there has been a sharper increase in the number of research workers in immunology over the last two decades than in any other scientific discipline.

Applied immunology, plus the intangibles we lump together as the rising standard of living, has virtually rid the world of smallpox, yellow fever, diphtheria and poliomyelitis and has helped in many other fields. Its prestige lingers on as the major tool of preventive medicine but, as one whose first immunological paper was published more than 50 years ago, I have seen a complete switch in the contemporary importance of immunology—but not a diminution.

Immunology today is a science in its own right. The enthusiasm of younger workers, like the authors of this book, is primarily directed toward understanding; medical applications of the new knowledge will be wholeheartedly welcomed but they are not central. For me, and to some extent all of us in immunology, the excitement is in the lead that our subject is giving toward a real understanding of the form and strategy of living process. Thanks to the *recognisability* of the significant molecules, antibody, antigen and the like, we have been able to apply the new techniques of molecular biology to the elucidation of one of the essential bodily functions. We are leading the field, for nowhere else have genetics, biochemistry and every other basic science that can help, been so effectively applied to living function. It is the first step toward a sophisticated understanding of what we are and how we became so.

This book is basically an introduction to the techniques and ideas on which immunology is based; to one who grew up with the older, predominantly medical approach, the new version can be sensed everywhere in the authors' approach.

I wish them every success.

F.M. BURNET
Basel, Switzerland
1976

ACKNOWLEDGEMENTS

Immunology has certainly changed since the first edition of *Practical Immunology*. Then laboratory workers had to produce virtually all their own antisera and much of the apparatus as well. Now the majority of reagents are bought ready made with appropriate fluorescent or enzyme labels attached. It was our policy from the start that the book should be complete, so that a technique could be performed without reference to numerous other texts. This has become increasingly difficult as each laboratory has its own preferred make of machine with associated reagents.

We have decided to continue with our original aim of assuming only basic equipment—such as might be available for a class practical. There are instructions for making reagents from first principles, to take account that not everyone using this book will have access to either the equipment or the funds to purchase everything to order.

Each previous edition has grown in size but we have been ruthless in cutting the length for this edition while including much new material. It is our firm intention that this should be an easily carried, working guide for undergraduates and research students. There are other reference tomes for the library shelf. With this edition Leslie Hudson has left for pastures new, and Frank Hay is delighted to welcome instead Olwyn Westwood, who has been extremely busy amassing new material. Paul Nelson has been most helpful in going through the completed text with us over several sessions and has supplied us with further useful material.

Immunology has become a very wide field and we have been grateful to other colleagues, particularly members of the immunology web news group, who helped us in the choice of methods to be included. Also, we are grateful to those who took the trouble to look through the draft manuscript, including Neville Punchard and Brian Ellis, who were most helpful in error trapping. Terry Poulton, Andy Soltys and Emma Frears were generous suppliers of help and advice. Special thanks are due to all our friends at Blackwell Science, especially Andrew Robinson, Fiona Goodgame and Karen Moore.

There are more references to the literature in this edition to guide the reader further, together with some key web sites. We have also set up a web site at http://www.sghms.ac.uk/depts/immunology/frankhay to maintain updates to methods. Please check the site to look for any modifications and do send us your tips and suggestions so that we can make them available for the benefit of other immunologists.

It is our hope that experienced immunologists, students and their lecturers alike will find this text useful, and we look forward to helpful discussions via the World Wide Web.

1 Isolation and structure of immunoglobulins

The following characteristics of the immunoglobulin classes can be used for their isolation and fractionation:

- solubility in aqueous solution;
- molecular size;
- electrostatic density;
- isoelectric point; and
- affinity for other molecules, e.g. lectins.

1.1 Fractionation by solubility

The relative solubility of proteins in pure water, ethanol or various salt solutions may be used as a basic fractionation technique. Serum may be separated into its euglobulin (insoluble) and pseudoglobulin (soluble) fractions by dialysis against distilled water. Although this is often used as the first step in the purification of immunoglobulin M (IgM), the euglobulin fraction is always contaminated with some immunoglobulin G (IgG).

1.1.1 Euglobulin precipitation to prepare IgM

MATERIALS

Sample: *either* monoclonal antibody culture supernatant of known immunoglobulin (IgM) *or*
30 ml serum derived from a subject who has fasted overnight (around 50 ml of whole blood)
Dialysis membrane tubing
Distilled water
Sephacryl S-200 HR in a column (100 × 2.5 cm) (see Appendix B.1.2)
0.1 M borate buffer, pH 7.4
UV spectrophotometer

Preparation of serum sample from whole blood

METHOD

1. Collect blood by venesection and allow to clot in a glass container without anticoagulant.
2. Once the clot has formed, separate the serum from the clotted cells by centrifugation at 1000 *g* for 15 min.
3. Transfer the serum (straw-coloured supernatant) to a suitable container, then proceed to isolation of the immunoglobulins.

Preparation of monoclonal antibody culture supernatant

METHOD

1. Centrifuge the sample at 10 000 *g* for 30 min at 4°C.
2. Save the supernatant and discard the cell debris, then proceed to next section.

Isolation of the immunoglobulins

METHOD

1. Secure one end of the dialysis tubing and decant in the spun supernatant or serum.
2. Dialyse against water to a volume that is 100 times the sample volume.
3. Collect the dialysed supernatant into a suitable test tube and centrifuge at 15 000 *g* for 60 min at 4°C.
4. Discard the supernatant.
5. Dissolve the pellet in 5 ml of borate buffer.
6. Prepare a column (100 × 2.5 cm) with Sephacryl S-200 HR and equilibrate with borate buffer.
7. Load the dialysed supernatant, and allow flow into the column.
8. Elute immunoglobulin with borate buffer, collecting 1 ml fractions, detecting the peaks by UV spectroscopy at 280 nm (IgM is eluted as the first peak).
9. Adjust the IgM to between 1 and 5 mg/ml and store at either 4°C or –70°C. See Appendix B.5.1 (methods for estimation of protein concentration).

TECHNICAL NOTES

- Increasing salt concentration of the medium leads to interference with the interaction of water molecules and the charged polar groups on protein molecules, i.e. rendering them less hydrophilic. This allows a greater hydrophobic interaction between protein molecules and they eventually become insoluble.
- The culture supernatant should contain around 1–50 mg/ml, therefore 500–1000 ml is required for a decent yield of IgM.
- The salt concentration at which each protein precipitates is different, but between closely related molecules such as immunoglobulins, the difference is not sufficiently great to give a precipitate with high-grade purity. However, it is often useful: (a) as a first step in isolation procedures as many serum proteins, e.g. albumin, will remain in solution when immunoglobulins are precipitated; and (b) for concentration of immunoglobulins from dilute solution.

1.1.2 Ammonium sulphate precipitation

Ammonium sulphate precipitation is a widely used for the preparation of a crude immunoglobulin fraction from whole serum. The use of ammonium rather than sodium sulphate as the precipitating salt offers the advantage of a high solubility that is only minimally dependent on temperature:

$(NH_4)_2SO_4$ ~ 3% variation between 0°C and 25°C;

Na_2SO_4 5 × more soluble at 25°C than at 0°C.

Relatively 'pure' IgG may be rapidly prepared by precipitation at a 33.3% saturation of ammonium sulphate. A higher yield of IgG at lower purity (i.e. containing other classes of immunoglobulin) is obtained at 50% saturation. However, smaller fragments of the molecule require higher salt concentrations for precipitation.

Preparation of serum immunoglobulin

MATERIALS AND EQUIPMENT

Ammonium sulphate
Dilute ammonia solution
Serum
UV spectrophotometer
0.14 M sodium chloride solution (saline)
Phosphate-buffered saline (PBS)

METHOD

1. Dissolve 1000 g ammonium sulphate in 1000 ml distilled water at 50°C, allow to stand overnight at room temperature and adjust to pH 7.2 with dilute ammonia solution.
2. Dilute 1 part serum with 2 parts saline and add an equal volume of saturated ammonium sulphate solution (prepared in step 1) to a final concentration of 45% saturated v/v.
3. Stir at room temperature for 30 min.
4. Centrifuge off precipitate (1000 *g* for 15 min at 4°C).
5. Wash precipitate with 45% saturated ammonium sulphate and recentrifuge.
6. Redissolve the precipitate in the same volume of PBS as the original serum.
7. Centrifuge to remove any insoluble material.
8. Reprecipitate the immunoglobulin using a final concentration of 40% saturated ammonium sulphate.
9. Centrifuge off the precipitate and wash with 40% saturated ammonium sulphate.
10. After centrifuging the washed precipitate, redissolve in a minimum volume of PBS.
11. Dialyse the immunoglobulins against five changes of PBS at 4°C (typically five changes of 1 litre). Centrifuge to remove any precipitate.
12. Prepare a 1 : 20 dilution of the immunoglobulins in PBS and determine the absorbance at 280 nm using a UV spectrophotometer. (Note the use of UV spectrophotometer requires pure immunoglobulin, as the technique is based on absorbance of UV light by aromatic amino acid residues in the protein.)

Calculation of protein content

At 280 nm, an absorbance of 1.0 (1-cm cuvette) is equivalent to an immunoglobulin concentration of 0.74 mg/ml.

Example:

if absorbance of sample diluted 1 : 20 = 0.95

immunoglobulin concentration = $0.95 \times 0.74 \times 20$

$= 14.1$ mg/ml.

TECHNICAL NOTES

- Use blood from a person who has fasted overnight as this has a low lipid content.
- Calculation of volume of saturated solution required to achieve a required concentration of ammonium sulphate:

$$V_r = \frac{100(S_f - S_i)}{1 - S_f}$$

where V_r is volume of saturated solution (ml) to be added per 100 ml volume of protein solution, S_f is final saturation (fraction, not percentage) and S_i is initial saturation (fraction, not percentage).

To minimize excessive volumes of solution when working in bulk, add solid ammonium sulphate according to the nomogram on the front inside cover.

- Determination of protein concentration by UV spectrophotometry is accurate down to about 0.05 mg/ml (see Technical note below).
- Use of protein solutions containing residual ammonium sulphate can interfere with some of the chemical reactions described in this book. It is good practice to test for residual ammonium sulphate by adding 1 drop of dialysate to 0.5 ml acidified barium chloride solution (use 1 M HCl to acidify a 10 mg/ml solution of barium chloride in water). If a precipitate forms, continue the dialysis of the protein solution.
- The extinction coefficient varies depending on the species. The figures quoted in Appendix B are for human immunoglobulins and provide a reasonable guide for immunoglobulins from other species. However, it is important to know that the UV absorption is dependent on the proportion of aromatic amino acids such as tryptophan. Polyclonal immunoglobulins will have an average content of these amino acids, but monoclonal antibodies are likely to give aberrant results owing to their unique composition.

Purification of mouse monoclonal antibodies

MATERIALS AND EQUIPMENT

Monoclonal antibody supernatant
Saturated ammonium sulphate solution, pH 7.2 (45–50% final saturation)
UV spectrophotometer

METHOD

1 Collect the monoclonal antibody supernatant and remove any contaminating cells by centrifuging at 10 000 *g* for 30 min at either 4°C or room temperature.
2 Precipitate the immunoglobulin with ammonium sulphate as described above in Section 1.1.2, using 40–50% saturated solution depending on purity required.
After dialysis determine the protein content of the solution using the following conversion factor: at 280 nm, absorbance of 1.0 (in a 1-cm cuvette) = 0.69 mg/ml immunoglobulin.

Rapid concentration of immunoglobulins

After column chromatography, samples are often recovered in dilute solution in large volumes of buffer. It is important to concentrate these rapidly as denaturation occurs in dilute solution. Ammonium sulphate precipitation is useful, using the solid salt to limit the total working volume of solution (nomogram, front inside cover).

The method described below is suitable for:

- light chains (see Section 1.6);
- Fab regions (see Section 1.7);
- preparing Bence-Jones proteins from the urine of patients with multiple myeloma.

MATERIALS

Material for concentration, for example:
Fab or light chains from column chromatography;
urine from patient with multiple myeloma;
urine from a mouse with a transplanted mineral oil-induced plasmacytoma or hybridoma
Solid ammonium sulphate
Phosphate-buffered saline (PBS)

METHOD

Steps 1–2 are for urine samples only; otherwise start at step 3.

1 Dialyse the urine against cold, running tap water for 24 h to remove inorganic salts and urea.
2 Centrifuge at 1000 *g* for 15 min to remove any insoluble material.
3 Adjust to pH 5.5 (salt precipitation is most effective at the isoelectric point of the protein required).
4 Add solid ammonium sulphate to 75% saturation. At 25°C, 575 g solid ammonium sulphate is required for 1000 ml of solution (see also nomogram, front inside cover). Add the salt slowly with stirring, otherwise it will form lumps bound up with protein that are very difficult to dissolve.
5 When all the salt has been added, stir for 1 h at room temperature to equilibrate.
6 Centrifuge at 1000 *g* for 15 min and discard the supernatant. (Take care to wash any salt off the rotor head or corrosion will occur.)
7 Redissolve the precipitate in PBS.

TECHNICAL NOTES

- Ammonium sulphate precipitation is often used to prepare crude γ-globulin fractions from whole serum. For many applications this may provide protein of sufficient purity, but even if highly purified material is required, salt precipitation may provide a useful first step in the isolation procedure.
- The redissolved precipitates still containing residual ammonium sulphate can be stored at 4°C or dialysed against the appropriate buffer system before use.

Combined ammonium sulphate and polyethylene glycol precipitation of IgM

Euglobulin precipitation can produce pure IgM but tends to give low yields, but it can be of use when a source rich in IgM is available, e.g. Waldenström's macroglobulinaemia serum.

When using polyethylene glycol precipitation of serum proteins it is necessary to remove lipid, e.g. by adsorption to silicon dioxide.

Tatum (1993) developed a method involving:

- low strength ammonium sulphate precipitation to remove lipids and fibrinogen (e.g. useful for plasma samples);
- high strength ammonium sulphate to isolate immunoglobulins;
- subsequent separation of IgM with polyethylene glycol.

MATERIALS AND EQUIPMENT

Plasma or serum
Saturated ammonium sulphate (SAS)
Polyethylene glycol 6000 (PEG-6000), 24% w/v in distilled water
Phosphate-buffered saline (PBS)
Distilled water
Centrifuge
Conductivity meter
pH meter
Dialysis tubing
1 M phosphoric acid

METHOD

1 Add 42 ml of SAS to 100 ml plasma with gentle stirring over 5 min.
2 Continue stirring at room temperature for 30 min.
3 Centrifuge for 20 min at 4000 *g*.
4 Discard pellet.
5 Add a further 50 ml SAS to the supernatant with stirring over 5 min.
6 Continue stirring at room temperature for 30 min.
7 Centrifuge for 30 min at 4000 *g*.
8 Remove as much supernatant as possible.
9 Resuspend the precipitate in 100 ml 50% SAS and stir gently for 5 min at room temperature.
10 Centrifuge for 20 min at 4000 *g*.
11 Remove as much supernatant as possible and redissolve the precipitate by the slow addition of a minimal volume of distilled water.
12 Adjust the conductivity to 80 mÙ$^{-1}$/cm^3.

Continued

13 Adjust the pH to 6.5–7.0 with 1 M phosphoric acid.
14 Add 1 volume 24% PEG-6000 for every 3 volumes of protein solution over 5 min with gentle stirring.
15 Continue stirring for 30 min.
16 Centrifuge at 4000 *g*.
17 Remove as much supernatant as possible and redissolve pellet in 5–10 ml PBS (or other desired buffer).
18 Dialyse to remove residual PEG and ammonium sulphate.

TECHNICAL NOTES

- The yield can be expected to be about 60–80% with a purity of over 90%, the major contaminants being IgG and IgA. These may be removed by gel filtration (Fig. 1.1; see Appendix B.1.2).
- Increasing the final PEG concentration from 6 to 7.5% will increase the yield of IgM at the expense of greater IgG contamination.
- Individual monoclonal antibodies, e.g. from patients with Waldenström's macroglobulinaemia, may need optimization of the method to get maximum yield. Lowering the temperature of PEG precipitation to 4°C can be helpful.

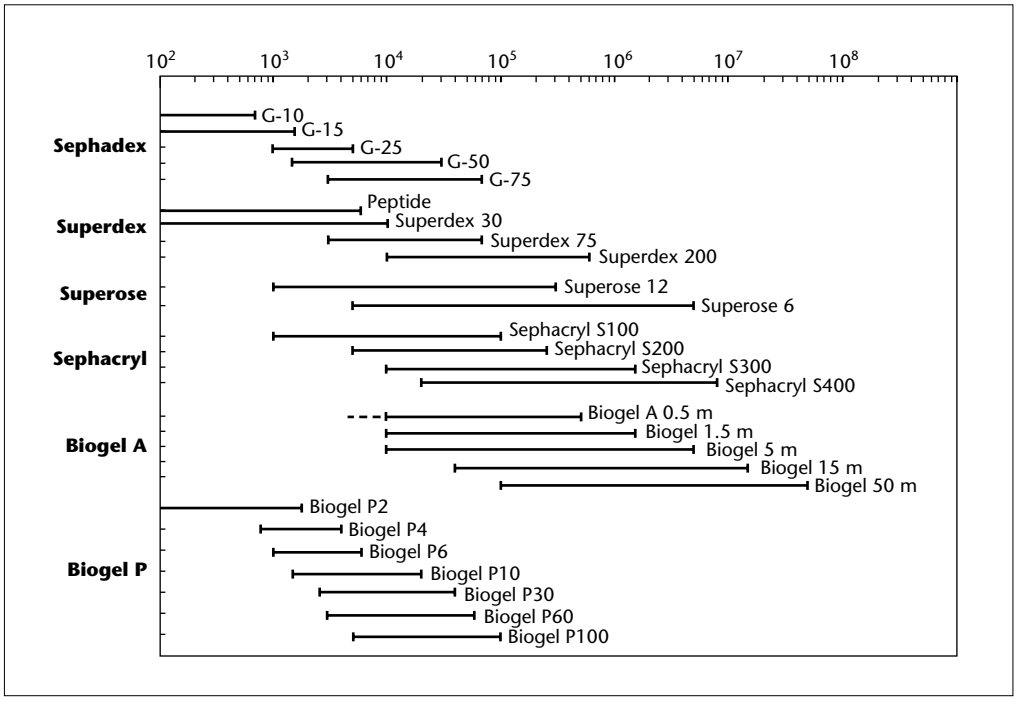


Fig. 1.1 Effective fractionation ranges for gel filtration media.
Sephadex G-10, G-50 and G-75: available in a range of bead sizes. The finest beads give better resolution but at the expense of slower flow rates.
Superdex Peptide and 30–200: prepacked columns.
Superose 6 and 12:
Sephacryl S100–S400:
Biogel A 0.5m–50m: for high resolution, narrow range fractionation.
Biogel P2–100: for small proteins and peptides.

1.2 Ultracentrifugation

Analytical and preparative ultracentrifugation have been widely applied in immunochemistry, for both molecular weight determinations and isolation procedures.

Preparative ultracentrifugation in sucrose density gradients is useful for the isolation of chicken IgM. Chicken IgM cannot be easily isolated by gel filtration as the IgG readily forms soluble aggregates, so appears within the excluded fraction of Sephacryl S-200 as a major contaminant of the IgM. However, the difference in size between the IgG dimers and the IgM is still sufficiently great to allow good resolution in the ultracentrifuge.

A detailed treatment of the basic techniques available, for example rate separation and isopycnic separation, both with and without a density gradient, is beyond the range of this book (see Lechner 1994).

1.3 Ion-exchange chromatography

Ion-exchange chromatography is an extremely useful method for the separation of proteins and the isolation of immunoglobulins. Proteins are bound electrostatically onto an ion-exchange matrix bearing an opposite charge. The degree to which a protein binds depends upon its charge density. Proteins are then eluted differentially by:

(a) increasing the ionic strength of the medium. As the concentration of buffer ions is increased they compete with the proteins for the charged groups upon the ion exchanger;
(b) alteration of the pH. As the pH of the buffer approaches the isoelectric point of each protein, the net charge becomes zero and so the protein no longer binds to the ion exchanger.

Both cation (e.g. carboxymethyl (CM)–cellulose) and anion exchangers (e.g. diethylaminoethyl (DEAE)–cellulose) are available. DEAE is used more widely for the fractionation of serum proteins. Cellulose remains the favoured support for the diethylaminoethyl group. Various forms are available to suit particular applications, and high-pressure liquid chromatography columns are available for analytical work.

1.3.1 Batch preparation of rabbit IgG with DEAE–cellulose

DEAE–cellulose and other ion exchangers can be used in columns or in batches. The batch technique is useful when large volumes of serum must be processed under standardized conditions. DEAE–cellulose is equilibrated under conditions of pH and ionic strength which allow all the serum proteins to bind except IgG. The serum must be pre-equilibrated to the same pH and ionic strength as the DEAE–cellulose, then simply stirred with the cellulose prior to recovering the supernatant containing IgG.

However, it should be noted that although this method is suitable for rabbit IgG, DEAE is not nearly as efficient for human IgG and so a gradient separation will be described for the latter.

Preparation of DE52 cellulose (DEAE)

MATERIALS

Diethylaminoethyl (DEAE)–cellulose DE52 (Whatman)
0.01 M phosphate buffer, pH 8.0
1.0 M HCl

METHOD

1. Place 100 g DE52 in a 1-l flask and add 550 ml 0.01 M phosphate buffer, pH 8.0.
2. Titrate the mixture back to pH 8.0 by adding 1.0 M HCl.
3. Leave the slurry to settle for 30 min, then remove the supernatant with any fines it may contain. Resuspend the cellulose in enough phosphate buffer to fill the flask.
4. Repeat this cycle of settling, decantation and resuspension twice.
5. Pour the slurry into a Buchner funnel containing two layers of Whatman no. 1 filter paper. Suck the cellulose 'dry' for 30 s to leave a damp cake of cellulose.

Preparation of IgG

The degree of purity of IgG is governed by the ratio of ion exchanger to serum; Fig. 1.2 illustrates the problems involved. For high purity, more cellulose is added but this leads to losses of IgG through binding to the ion exchanger. The precise proportions used depend upon the required purity of the IgG. Reasonable purity (about 96%) and good yield (about 70%) are obtained using 5 g (wet weight) cellulose for every ml of serum.

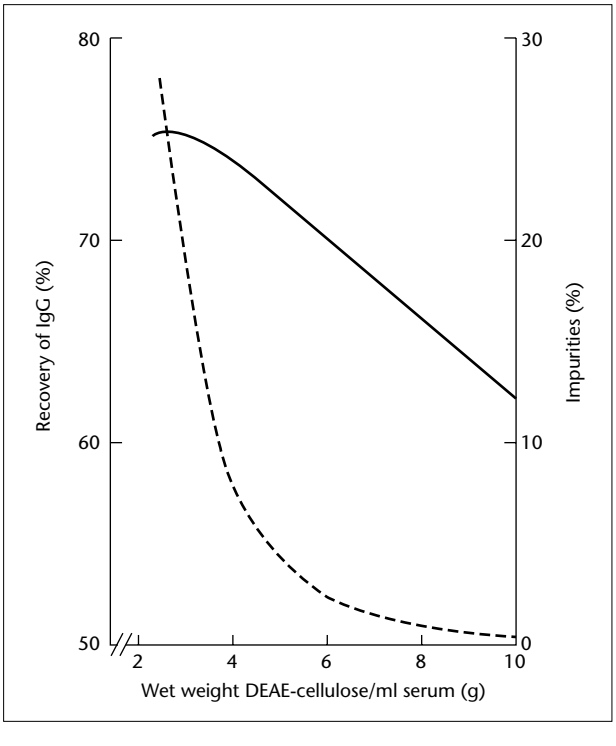


Fig. 1.2 Relationship of purity (dashed line) and yield (solid line) of IgG to the amount of ion exchanger used per ml of serum. This illustrates a universal rule of all protein purifications that the higher one tries to make the yield the lower will be the purity.

METHOD

1. Weigh the cellulose into a beaker; for every 10 ml serum use 50 g wet weight of cellulose. Mix 10 ml serum with 30 ml distilled water, to lower its ionic strength, and add to the cellulose at 4°C.
2. To equilibrate stir thoroughly every 10 min for 1 h at 4°C.
3. Pour the slurry onto a Buchner funnel and suck through the supernatant; this contains the required IgG. Rinse the cellulose with 3 volumes of 20 ml 0.01 M phosphate buffer, pH 8.0.
4. Collect and combine all the filtrates.

Examination of IgG preparation

1. If a determination of yield is required, then the IgG content in the original serum and the filtrate may be measured immunologically; e.g. using either rate nephelometry (see Section 3.6) or radial immunodiffusion (see Section 3.4).
2. The purity of the preparation may be determined by comparing the IgG content (measured above) of the filtrate with its total protein content (determined by UV spectrometry).
3. Use SDS-PAGE or immunoelectrophoresis against anti-whole rabbit serum to identify the main contaminants of the IgG (see also Appendix B.2.1 and Sections 3.2–3.9).

1.3.2 Preparation of IgG with an ionic strength gradient

For maximum yield and purity a column technique using gradient elution is preferred for the preparation of IgG of any species. For human and mouse IgG gradient elution is essential. Initially, buffering conditions are adjusted such that virtually all the serum proteins bind to the ion exchanger. Proteins are then eluted sequentially by gradually increasing the ionic strength of the buffer running through the column.

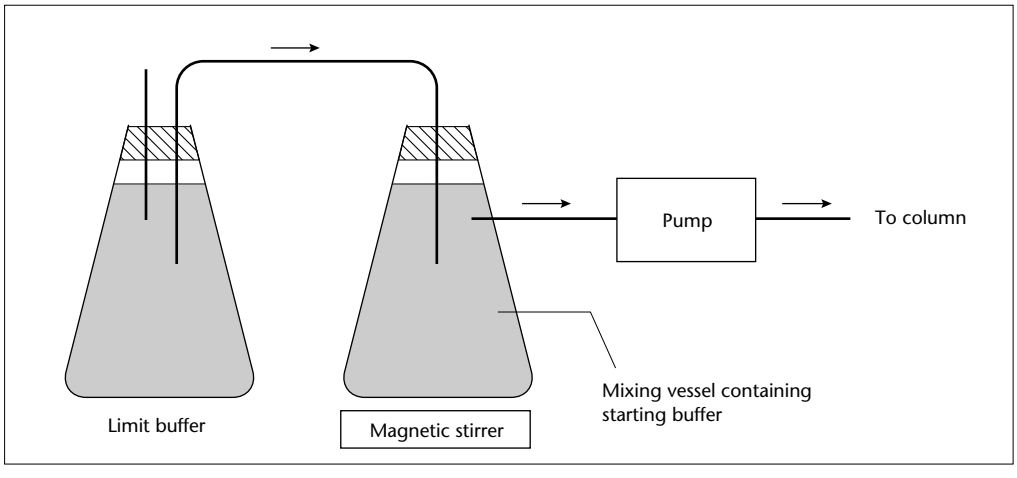


Fig. 1.3 Simple apparatus for the production of an exponential gradient. When the buffer concentration in the limit vessel is greater than the initial concentration in the mixing vessel a convex gradient is produced. Sophisticated pumps are now supplied for fast performance liquid chromatography (FPLC) and high performance liquid chromatography (HPLC) systems, with microprocessor controllers, which allow the programming of an almost limitless range of gradients. Arrows indicate the direction of flow.

MATERIALS AND EQUIPMENT

Serum sample

Diethylaminoethyl (DEAE)–cellulose DE52 (Whatman)

Column and fraction collection apparatus (a short wide column is preferable; e.g. 25 × 3.3 cm; see Appendix B, Fig. B.1)

Gradient device (commercially available, or constructed as in Fig. 1.3)

Conductivity meter

Phosphate buffers, pH 8.0, 0.005 M and 0.3 M

METHOD

A Equilibration of ion exchanger

1. Place the ion exchanger in a beaker—use 2–5 g (wet weight) DE52 for every 1 ml of serum.
2. Add the basic component to the phosphate buffer (0.5 M disodium hydrogen phosphate) until the pH reaches 8.0.
3. Add 0.005 M phosphate buffer, pH 8.0. There should be 6 ml buffer for every 1 g of wet ion exchanger.
4. Disperse the cellulose and pour into a measuring cylinder and allow to settle (settling time [min] = 2 × height of the slurry [cm]). Remove the supernatant that contains cellulose 'fines'; these may block the column.
5. Add a volume of 0.005 M buffer equal to half the volume of settled cellulose and resuspend.
6. Pour the slurry into the column with the flow-control valve open.
7. Pack the column by pumping 0.005 M, pH 8.0, phosphate buffer through at 45 ml/h for each cm^2 internal cross-section.
8. Monitor the buffer effluent with a conductivity meter. When the ionic strength of the effluent is the same as that of the original buffer, the ion exchanger is equilibrated. If a meter is not available, pass 2–3 l of buffer through the column.

B Running the column

1. Dialyse the sample against the starting buffer (0.005 M, pH 8.0 phosphate buffer).
2. Centrifuge the sample. (Some protein will precipitate at this low ionic strength.)
3. Apply the serum to the column and pump through the starting buffer (about 60–100 ml/h). Monitor the effluent for protein. Most of the proteins should bind to the ion exchanger.
4. Elute the proteins with a gradient of increasing ionic strength (see below). Collect fractions of approximately 5 ml.

TECHNICAL NOTE

If a high concentration of protein is detected in the column effluent prior to the application of the ionic strength gradient, either (a) the ion exchanger or serum was not fully equilibrated or (b) the absorbing capacity of the cellulose has been exceeded.

Ionic strength gradient

Gradients of varying shapes are used for different purposes. A great variety of commercial gradient-forming equipment is available, ranging from simple devices which are essentially two chambers joined together (similar to Fig. 1.3), to sophisticated electronic systems in which the rate of advance of the gradient is controlled by a monitor for protein in the column effluent. In

this system a discontinuous gradient can be formed automatically and greatly increases the resolution of ion-exchange chromatography.

A continuous exponential gradient may be produced as shown in Fig. 1.3. The limit buffer enters the mixing vessel at the same rate as the buffer is pumped onto the column. The gradient is established according to the following equation:

$$C_m = C_l - (C_l - C_0)e^{-v/v_m}$$

where C_m is concentration in mixing vessel, C_l is concentration in limit vessel, C_0 is initial concentration in mixing vessel, v is volume removed from mixing vessel and v_m is volume of the mixing vessel.

For ease of calculation this equation may be rewritten as:

$$2.303 \cdot \log\frac{C_l - C_m}{C_l - C_0} = \frac{-v}{v_m}$$

When $C_l > C_0$ the gradient is convex; when $C_l < C_0$ the gradient is concave. (The latter is used for density gradient formation not ion-exchange chromatography: the highest ionic strength buffer will emerge first and elute everything off the column.)

Linear gradients may be established using an open mixing vessel (Fig. 1.4a) or by means of a multichannel pump as shown in Fig. 1.4(b). In this case the equation for the gradient is:

$$C_m = C_0 + (C_l - C_0)\frac{v}{2v_0}$$

where v_0 is initial volume of buffer in the mixing vessel and other symbols are as in the equation for exponential gradient above.

Distribution of serum proteins

Assuming that the ion exchanger has not been overloaded with protein the first peak should contain only IgG. This is the only pure protein that can be isolated under these conditions of pH and buffer molarity; the remaining peaks contain several proteins. Beta-lipoproteins, haptoglobin and α_2-macroglobulin will contaminate the IgA and IgM fractions.

Regeneration of the ion exchanger

1. Remove the ion exchanger from the column by washing out with distilled water.
2. Add 0.1 M HCl (0.5–1 × bed volume of cellulose).
3. Place on Buchner funnel and rinse through with distilled water.
4. Add 0.1 M NaOH (volume equivalent to the HCl used) and then rinse through with distilled water.
5. Wash through with full strength buffer and then re-equilibrate with low ionic strength buffer.

TECHNICAL NOTE

To store cellulose ion exchangers add chlorhexidine to a concentration of 0.002% for anion exchangers and sodium azide to 0.02% for cation exchangers.

Note: Azide is a dangerous chemical—do not discard down the sink.

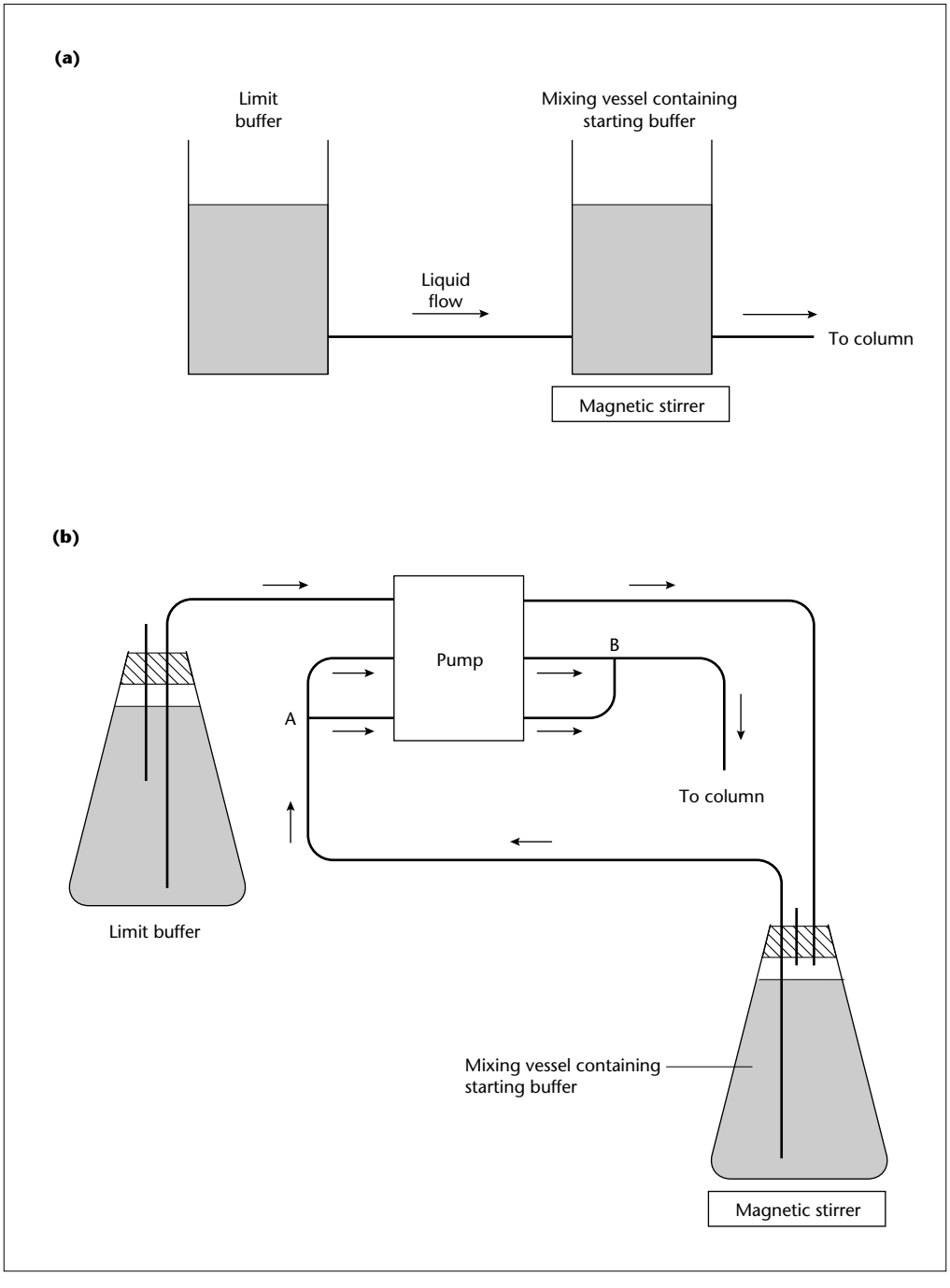


Fig. 1.4 (a) **Formation of a linear gradient using an open mixing vessel.** The effective volume of the mixing chamber reduces as the gradient is formed. (**b**) The production of a linear gradient by means of a multichannel pump. Tubing from B to the column must be of sufficient internal diameter to take twice the flow rate in the rest of the system. Arrows indicate direction of flow. A and B are 'h'-type functions.

1.3.3 Mass production and mini-column ion-exchange chromatography

Conditions for running ion-exchange columns are less critical than for gel filtration; it is therefore possible to set up large numbers of mini-columns in cheap apparatus such as disposable syringe barrels. The conditions are sufficiently reproducible that fixed volumes of cellulose, serum and buffer give reproducible preparations of IgG and are technically so simple that up to 20 syringe columns in a rack with gravity flow may be run simultaneously. The following procedure will rapidly give very pure IgG.

MATERIALS AND EQUIPMENT

Serum (human)
Saturated ammonium sulphate
Phosphate buffers 0.02 M and 0.2 M, pH 7.2
1 M potassium chloride in 0.02 M phosphate buffer, pH 7.2
Disposable syringe with a central nozzle (e.g. 10-ml hypodermic syringe)
Diethylaminoethyl (DEAE)–cellulose DE52
Glass or nylon wool, or sintered plastic disc

Caution: Wear gloves when handling glass wool.

METHOD

1. Add 1 ml saturated ammonium sulphate dropwise to 2 ml human serum to give a 33% saturation. Stir for 30 min. Precipitating the serum with ammonium sulphate eliminates much of the material which would otherwise bind to the ion exchanger and reduce its capacity.
2. Spin the precipitate at 1000 *g* for 15 min and resuspend the pellet in 40% saturated ammonium sulphate.
3. Stir for 10 min and then spin at 1000 *g* for 15 min.
4. Resuspend the pellet in 0.02 M phosphate buffer, pH 7.2.
5. Dialyse the sample against 0.02 M buffer overnight.
6. Block the outlet of a disposable syringe with a little glass or nylon wool or a sintered plastic disc.
7. Place 3 g (wet weight) of DEAE–cellulose in the syringe and wash through with 5 ml 0.02 M phosphate buffer containing 1 M KCl.
8. Wash the column with 20 ml 0.02 M phosphate buffer (without KCl).
9. Add the dialysed protein sample to the cellulose.
10. Elute the IgG with 15 ml 0.02 M phosphate buffer and collect 3 ml fractions.
11. Determine the absorbance at 280 nm of the fractions and pool those containing protein. These contain the IgG.
12. Calculate the yield of IgG using the extinction coefficient given in Appendix B.
13. Elute the bound protein from the column with 0.02 M phosphate buffer containing 1 M KCl.
14. Regenerate the DE52 column as in step 8 above.

TECHNICAL NOTE

Greater throughput efficiency may be achieved by combining the protein dialysis and ion-exchange media in the same column. Layer Sephadex G-25 on top of the DE52 cellulose

and equilibrate both as above. Filtration of the protein sample through the Sephadex G-25 will allow sample equilibration by buffer exchange prior to interaction with the DE52 cellulose. Using this procedure, many samples of highly purified IgG may be prepared during one working day.

1.3.4 QAE–Sephadex isolation of IgG

Quaternary aminoethyl (QAE)–Sephadex is a strongly basic anion exchanger that is particularly suitable for the column separation of proteins using pH gradient elution as the swelling of QAE–Sephadex is not affected by changes in pH. The advantage is that IgG may be prepared using a volatile buffer and freeze dried without prior salt removal. It is advisable to remove β-lipoproteins from the serum before chromatography, otherwise they may break through and contaminate the IgG.

MATERIALS AND EQUIPMENT

Human serum
Aerosil
Diamino ethane–acetic acid buffer, ionic strength 0.1, pH 7.0
Acetic acid–sodium acetate buffer, ionic strength 0.1, pH 4.0
Quaternary aminoethyl (QAE)–Sephadex A-50
Column and fraction collection apparatus
1.0 M sodium hydroxide
Polyethylene glycol 8000 (PEG-8000)
Dialysis membrane tubing
Centrifuge capable of 12 000 *g*
UV spectrophotometer

METHOD

1. Swell QAE–Sephadex A-50 in the diamino ethane–acetic acid buffer. A bed volume of 20 ml of swollen gel is required per 10 ml serum.
2. Pack the gel into a suitable chromatography column and equilibrate with the diamino ethane–acetic acid buffer.
3. Remove β-lipoprotein from the serum by adding 0.2 *g* Aerosil to 10 ml serum and stir at room temperature for 4 h.
4. Centrifuge the serum at 12 000 *g* for 30 min and remove the lipid layer.
5. Equilibrate the serum with the diamino ethane–acetic acid buffer by dialysis or column buffer exchange.
6. Dilute the equilibrated serum with an equal volume of diamino ethane–acetic acid buffer. (If column buffer exchange was used the sample will have already been diluted by passing through the column.)
7. Apply the sample to the column at a flow rate of 8 ml/cm^2/h and continue the elution with the diamino ethane–acetic acid buffer. IgG will come straight through the column while other proteins will be retained. Assess completion of the elution by monitoring the optical density (OD) of elute using a UV spectrophotometer.
8. Elute the other proteins with the acetate buffer, pH 4.0.

Continued on p. 16

9 Regenerate the column by running through two bed volumes of diamino ethane–acetic acid buffer.
10 Concentrate the IgG in the first peak to 1/10 volume as quickly as possible; e.g. using dialysis tubing and PEG-8000.
11 The concentrated sample may now be freeze dried without removing salt as the buffer is volatile.

TECHNICAL NOTES

- It is important to concentrate the sample prior to lyophilization otherwise an insoluble precipitate may form.
- The yield of IgG should be about 70% of the serum IgG.
- Conditions should be optimized when preparing immunoglobulins from other species.

1.4 Affinity techniques for immunoglobulins and other molecules

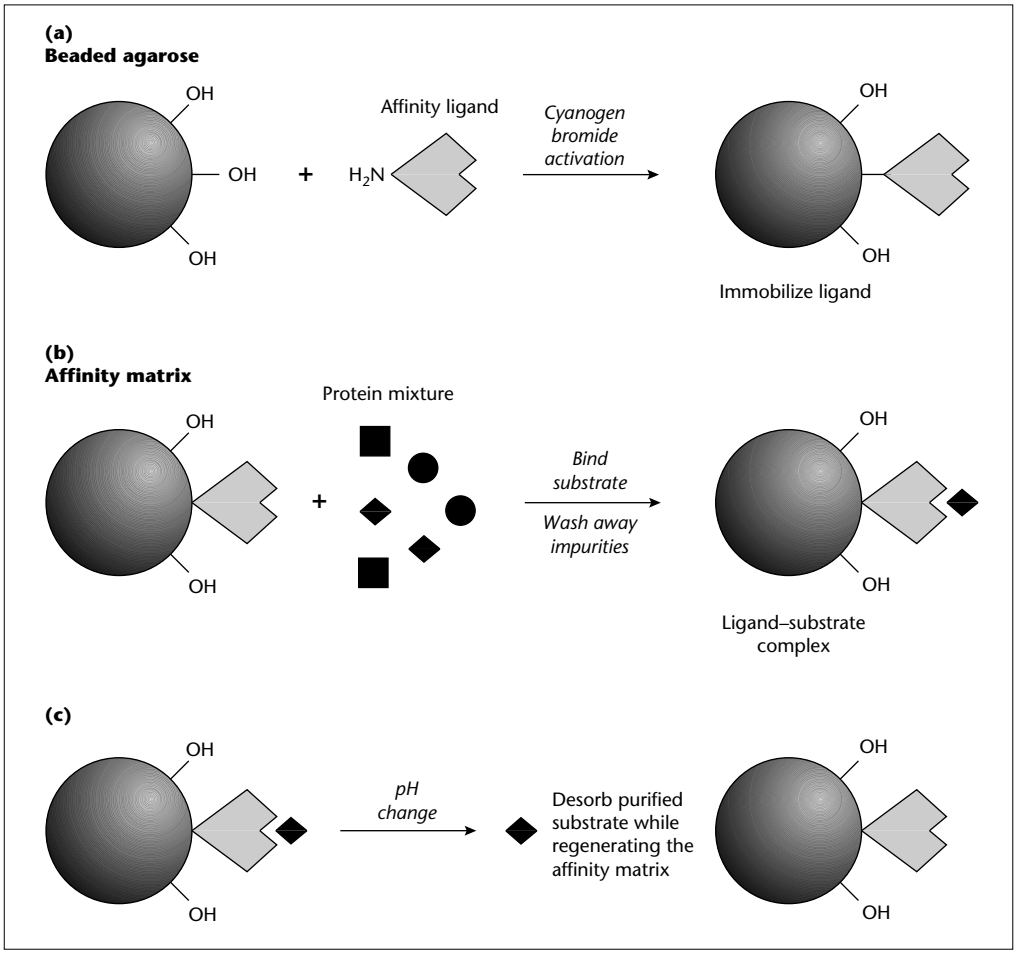
The series of techniques described in this chapter combine the two most sought-after attributes in any purification procedure: (i) large gains in purity in single-step procedures; and (ii) technical simplicity.

In affinity chromatography the technique (summarized in Fig. 1.5) is (a) achieved by the selection of an affinity ligand that shows strong, selective and reversible binding to the molecule being purified (in operational terms, the ligand's substrate) and (b) facilitated by the use of an insoluble (and, preferably, chemically inert) affinity matrix thus permitting rapid partitioning of the ligand and its substrate.

Axen, Porath and Ernbach introduced a general technique for affinity chromatography whereby molecules containing primary amino groups could be coupled to insoluble polysaccharide matrices activated by cyanogen bromide. This route of derivatization is still the most widely employed today, even though the matrix so formed has the disadvantage of charged isourea groups, leading to a bioselective matrix with ion-exchange properties, and unstable covalent bonds between the matrix and ligand, which are susceptible to nucleophilic attack.

Support matrices such as beaded agarose gels may be used. Commercially prepared agarose beads consist of linear chains of agarobiose units in which the ionic charge of the repeating

Fig. 1.5 (*opposite*) **Affinity chromatographic separation of substrate molecules.** (**a**) Preparation of the affinity matrix. Although we have chosen to illustrate cyanogen bromide activation of Sepharose, there is an enormous range of different solid supports and derivatization reactions available (see Further reading at end of chapter). The solid support should be chemically and biologically inert (before and after derivatization); it should have a large surface area and a physical form (e.g. beaded) that will permit a high flow rate; and its physical and chemical stability should not be affected by the conditions used for desorption (treatment with free ligand, chaotropic agents, agents which disrupt hydrogen bonding detergents, etc., or changes in pH and ionic strength). The derivatization reaction should result in an uncharged covalent bond between the ligand and solid support which is stable during both desorption and long-term storage. It should not inactivate the ligand! Sometimes the ligand is sterically hindered by the support, resulting in a low adsorptive capacity



(the theoretical upper limit of the affinity matrix may be calculated from the amount of ligand bound and the stoichiometry of the ligand–substrate interaction). This can be frequently overcome by the use of a 'spacer arm' between the support and ligand (see Further reading at end of chapter).

(**b**) Capture of the substrate molecules. Practical considerations are very important at this stage: e.g. the mixture containing the substrate should be in complete solution (this can be a particular problem with detergent-solubilized cells); the insolubilized ligand and substrate should have sufficient time to interact (do not run the columns too fast and recycle the column effluent several times); and the final washing of the column should be exhaustive to ensure that no unbound or weakly bound material is trapped in the interstices of the column.

(**c**) Desorption of the purified substrate. It is only rarely possible to desorb the substrate purely by competition with free ligand. Consider, for example, a relatively simple system such as the purification of anti-dinitrophenyl (anti-DNP) antibodies on a DNP–bovine serum albumin affinity column. Even when using very small molecules for free ligand competition, such as DNP–lysine, it is impossible to achieve a sufficiently high local concentration of free ligand, in the environment of the affinity matrix and anti-DNP binding site of the antibody, to be able to compete with the high-avidity multipoint interaction. Instead it is necessary to reduce or neutralize the forces of interaction originally responsible for capturing the bound substrate. A reduction of the interactive forces will sometimes permit the final release of the bound substrate molecules by free ligand competition; this brings an additional specific desorption step to the whole technique and so gives greater purity of product. The simultaneous desorption of substrate and regeneration of the matrix is a particularly appealing feature of affinity chromatography; the column need only be returned to the adsorptive conditions to start the whole process again.

1,3-linked β-D-galactopyranose and 2,4-linked 3,6-anhydro-α-galacto-pyranose moieties is removed by reduction with sodium borohydride under alkaline conditions.

As there are no natural covalent bonds between the linear polysaccharides, these are introduced by treatment with epi-chlorohydrin, improving the mechanical and chemical properties of the gel, thus permitting higher flow rates without compression of the gel bed and leading to improved stability at higher temperatures and in the presence of denaturing or chaotropic agents, etc. (Sepharose CL-4B is a commercially available gel with these physical and chemical properties.)

Matrix derivatization–cyanogen bromide activation

Cyanogen bromide reacts with the vicinal diols of agarose (also dextran and cellulose) to produce an activated matrix which will react with ligands (or spacer arms) containing unprotonated primary amines as summarized in Fig. 1.6. The isourea group is positively charged at physiological pH and can act as an ion-exchange matrix with negatively charged proteins.

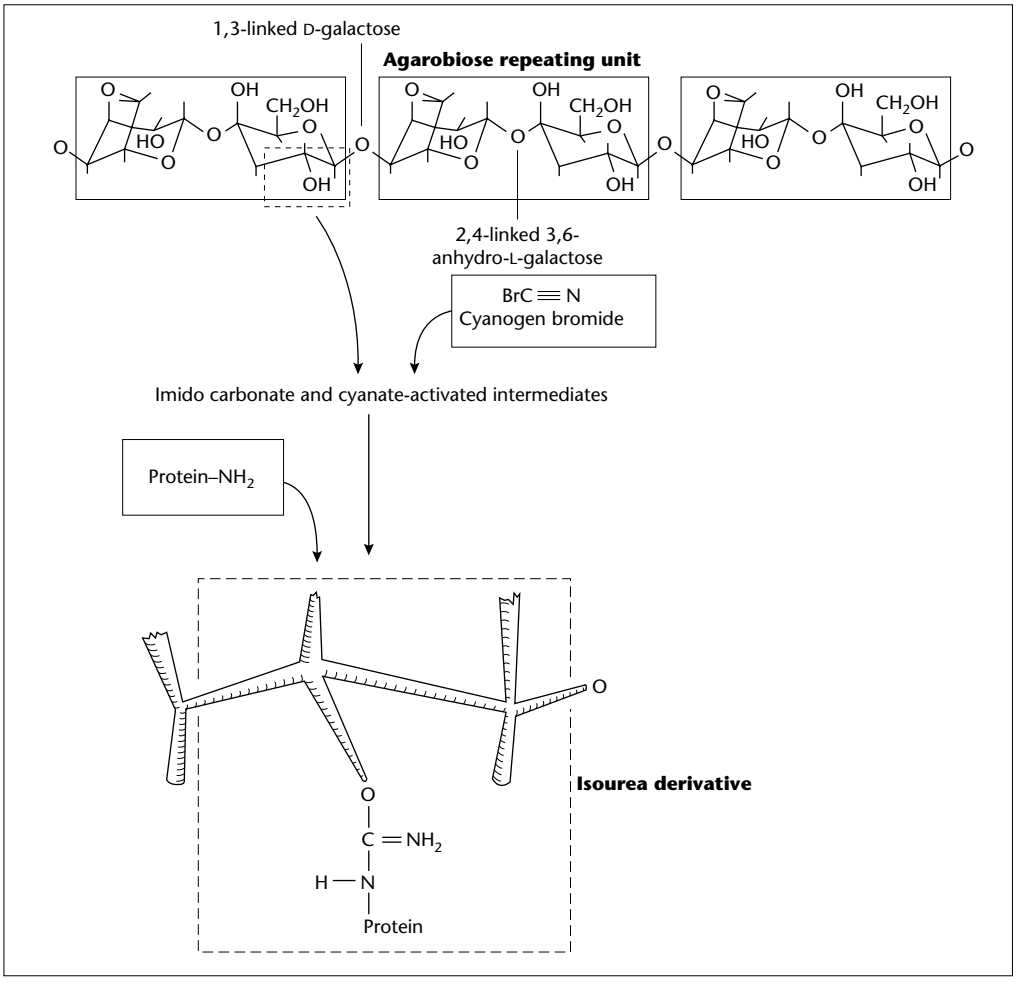


Fig. 1.6 Cyanogen bromide activation of agarose.

1.4.1 Preparation of immunoglobulin isotypes by affinity chromatography

Affinity chromatography may be used to purify immunoglobulin isotypes as an alternative to the physical chemical methods. The most obvious way to use affinity adsorption: prepare an insoluble antibody specific for the required isotype. However, this requires that the purified isotype first be available to prepare the antibody for immunosorption. Fortunately, immunoglobulins have affinity for a range of other molecules. For example:
IgG binds strongly to protein A, a cell-wall protein derived from *Staphylococcus aureus*;
IgM binds to mannan-binding protein;
IgA_1 binds to the lectin jacalin, and mouse IgD binds to *Griffonia simplicifolia* I lectin.

The above examples of affinity methods for isolating immunoglobulins largely (except for protein A and G) depend on the recognition of sugars. While particular sugars are associated with various immunoglobulin isotypes the relationship is not absolute and may be altered in disease. Using these methods always has some danger of contamination and yields will not be 100%.

1.4.2 Preparation of IgG on protein A–agarose

The IgG binding properties of protein A make affinity chromatography with protein A–agarose immunoadsorbents a very simple method for preparing IgG. IgG subclasses show differential binding: e.g. human IgG subclasses 1, 2 and 4 bind to protein A but IgG_3 does not.

MATERIALS

Human serum
Protein A–agarose, e.g. protein A–Sepharose CL-4B
Phosphate-buffered saline (PBS)
0.1 M glycine–HCl, pH 2.8
1 M sodium hydroxide or solid tris (hydroxymethyl)-aminomethane (Tris)

METHOD

1. Swell 1.5 g protein A–Sepharose CL-4B in 10 ml PBS for 1 h at room temperature and then pack it into a small chromatography column, e.g. a 10-ml disposable hypodermic syringe. Store and use this column at 4°C.
2. Dilute 10 ml human serum with an equal volume of PBS.
3. Filter the serum through the column at a flow rate of 30 ml/h.
4. Wash through unbound proteins with PBS until no more protein leaves the column (monitor the protein with a UV flow cell).
5. Elute the bound IgG with glycine–HCl buffer, pH 2.8.
6. Titrate the pH of the purified IgG solution to near neutrality with NaOH or solid Tris, and dialyse against PBS.
7. Regenerate the column by washing with 2 column bed volumes of PBS. Store the column at 4°C.

TECHNICAL NOTES

- The protein A content of the swollen gel is 2 mg/ml and the binding capacity for human IgG is approximately 25 mg/ml of packed gel.
- Small quantities of some types of IgM will bind to protein A. You should be aware of this possibility and monitor the IgG preparations if absolute purity is required. Remove the IgM by gel filtration.
- Protein G is also useful for preparing IgG. It has a slightly different range of subclass specificities and is particularly good for preparing rat IgG. Generally it has a high capacity for binding IgG and similar conditions may be used as for protein A isolation of IgG.

  As well as IgG, protein A binds to the V_H3 region of other immunoglobulin subclasses.

  Protein G shows greater specificity for IgG. Protein L, derived from *Peptostreptococcus magnus*, binds to immunoglobulins through interaction with κ light chains, particularly human κI, κIII and κIV and mouse κI. It therefore binds all immunoglobulin classes, but omits all antibodies with λ light chains. Protein L can be bound to agarose gels using cyanogen bromide and used as for protein A. (See De Chateau *et al*. (1993) which discusses the interaction between protein L and immunoglobulins of various mammalian species.)

1.4.3 Isolation of IgG subclasses using protein A–agarose

Although in both human and mouse the IgG subclasses differ markedly from each other in their biological properties, they are structurally very similar. This similarity has made it almost impossible to isolate single subclasses using physical chemical techniques. Fractionation of the IgG subclasses is possible using protein A affinity chromatography and pH gradient elution.

Isolation of mouse subclasses

IgG is common to mammalian species, but further evolution has occurred since subclasses of IgG are present in many animals but there is no clear relationship between subclasses in different species. IgG_1, IgG_2, IgG_3 and IgG_4 are found in humans; IgG_1, IgG_{2a}, IgG_{2b} and IgG_3 in mice; and IgG_1, IgG_{2a}, IgG_{2b} and IgG_{2c} in rats.

Mouse serum may be fractionated on protein A–agarose by:

1 allowing all the IgG to bind to the adsorbent; and then
2 eluting the separate subclasses with a stepped gradient of increasing acidity.

MATERIALS AND EQUIPMENT

Mouse serum
Protein A–Sepharose CL-4B
Phosphate-buffered saline (PBS)
0.1 M phosphate buffer, pH 8.0
0.1 M citrate buffers, pH 6.0, 5.5, 4.5, 3.5
1.0 M tris (hydroxymethyl)-aminomethane(Tris)–HCl buffers, pH 8.5, 9.0
Chromatography column or 10-ml disposable syringe
Antisera to the mouse IgG subclasses