IMMUNOCHEMISTRY IN PRACTICE

Alan Johnstone/Robin Thorpe

SECOND EDITION

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IMMUNOCHEMISTRY IN PRACTICE

FIRST EDITION

TO THE MEMORY OF

LAWRENCE EDWARD MOLE

SCIENTIST AND TOFF

SECOND EDITION

TO THE MEMORY OF

RODNEY ROBERT PORTER

Foreword

Immunology has been important in medicine since Jenner introduced his technique of vaccination to prevent smallpox infection nearly 200 years ago. It is only in recent years, however, that greatly increased understanding of the complex reactions responsible for immunity has given the opportunity to exploit these phenomena, both for the study of immune reactions themselves and also for the investigation of many other problems in biology.

The major feature of immune reactions is their specificity, and antibodies are reagents which can be prepared at will with specificities directed to almost any chemical grouping whether it be in a protein, carbohydrate, nucleic acid or a simpler organic compound. The introduction of monoclonal antibodies produced by fusion of antibody forming cells and transformed cells has made available entirely specific reagents against an apparently infinite range of substances. The application of these reagents to detect, estimate and isolate different molecules in complex biological fluids or on cell surfaces is revolutionizing much biological research. Monoclonal antibodies are becoming major diagnostic reagents and may soon find applications in therapy.

To exploit fully the specific reactions of these substances many techniques have to be mastered. They are often exacting and a thorough understanding is necessary for their effective use. In this book an excellent account is given of these diverse methods and I have no doubt that it will be most valuable for the rapidly increasing number of scientists from different fields who are taking up immunochemical techniques.

R. R. Porter

Preface

TO FIRST EDITION

Rapid progress in recent years has made many scientists increasingly aware of the potential role for immunology in many areas of research. Unfortunately the same progress has tended to widen the psychological gulf between immunology and other biological sciences, all of which would benefit from a freer interchange of experimental expertise. This book seeks to bridge that gulf by describing the preparation, characterization and application of immunoglobulins and antibodies, together with several analytical and preparative biochemical techniques.

Each procedure is described in detail for one pertinent application (as an illustrative example for research or practical classes), followed by comprehensive explanatory notes including modifications necessary for wider use. A basic knowledge of laboratory practice and a supply of routine laboratory apparatus and chemicals is assumed but beyond this full details are included in the description. The emphasis throughout is on practical aspects but sufficient theory is given to understand the principles of the techniques. A set of references is provided for wider theoretical discussion.

TO SECOND EDITION

Immunochemistry has advanced considerably during the five years since we wrote the first edition. This has been very good for the progress of basic science in the many diverse fields that use immunochemical techniques and also for applied uses in the biotechnology industry. However, the changes have been bad for a couple of lazy authors who thought that their first edition would serve for many years. We have revised and updated all the chapters with advances in handling and applying monoclonal antibodies at the forefront of the changes.

One of the saddest events of the last five years has been the death of Rodney Porter in September 1985. He carried out a large proportion of the basic research on which much of today's applied immunochemistry is based, but, more than that, he inspired many scientists by his incisiveness, integrity and humour combined with a brusque straightforwardness. After dedicating our first edition to one of his students, we would like to dedicate the second to him with many many thanks.

Acknowledgements

TO FIRST EDITION

This book was conceived with a great deal of support from Leslie Hudson. We are grateful to him for his continuing advice and encouragement throughout its progress towards birth.

We wish to thank the numerous colleagues who helped to shape the book with their discussion, advice on methodology or illustrations, in particular: Hansha Bhayani, Maryvonne Brasher, Caroline Bullock, Annette Ford, Jens Jensenius, Alison Mackay, Philip Minor, Sue Peach, Moises Spitz, Alan Williams, Gwyn Williams and John Wood. We are especially grateful to Professor Porter for writing the Foreword.

The completion of the work was due in large part to our wives, Caroline and Sue, who provided mental support and practical help (scientific and editorial) throughout.

Our thanks are also due to Kay Dorelli who completed the arduous task of preparing the typescript in record time.

TO SECOND EDITION

In addition to our colleagues who helped with the preparation of the first edition, we would like to thank all those who contributed to the revision by giving advice on methodology or illustrations, in particular: Chris Bird, Marion Callus, David Darling, Caroline Edmonds, Andy Gearing, Roy Harris, Jan Hawthorn, Leslie Hudson, Susan King, Ruth McNerney, John Murphy, Varsha Patel, Phil Robinson, Edith Sim, Lynne Trickett, Guy Whitley and David Winterbourne. In addition, we are grateful for the encouragement of many reviewers of the first edition and numerous scientists around the world who troubled to write or telephone with helpful comments and suggestions.

Like the first edition, this revision was greatly aided by our wives, Caroline and Sue, who have stayed with us for another five years and provided much editorial and scientific help. Thanks are also due to Elinor Johnstone who, at the age of 3½, has nearly learnt to sleep through the night!

Contents

1 Basic techniques

This chapter describes briefly some basic methods central to immunochemistry. They are referred to throughout the book. Some relevant immunological techniques are presented in Chapter 2.

1.1 DETECTION AND MEASUREMENT OF PROTEINS

The simplest method for measuring the concentration of protein in solution is by its absorption of ultra-violet (UV) light (Section 1.1.1). If the protein is pure its absolute concentration can be calculated from the value obtained. If the protein is not pure (e.g. in the eluant from column chromatography) the absorbance will give an approximate estimate of the total protein concentration. The method cannot be used at low protein concentrations (below about 0.05–0.1 mg/ml), in the presence of many substances which also absorb in the UV range (e.g. some buffers, nucleic acids and some lipids) or if the protein is in suspension, not solution (e.g. in membranes or large complexes). Colourimetric methods are more complicated and the samples taken are not recoverable (Section 1.1.2). However, they are usually more sensitive and the solutions used solubilize most proteins.

1.1.1 Ultra-violet absorption

Proteins absorb UV light with a maximum at approximately 280 nm, caused by tryptophan, tyrosine and (to a lesser extent) phenylalanine residues, and at lower wavelengths (215–230 nm) because of the polypeptide chain backbone. Absorbance at 280 nm varies for each protein, but the recorded extinction coefficients (i.e. the absorbance of a 1% solution of the protein in a 1 cm light path) for individual proteins (Table 1.1) allow the concentration of a pure protein to be calculated. The absorbance at lower wavelengths is directly related to the weight of polypeptide material and is usually considerably more sensitive than at 280 nm. However, many buffers and other molecules also absorb at these lower wavelengths (phosphate and tris buffers are acceptable but the preservative sodium azide absorbs strongly). Absorbance at 215 or 230 nm is useful for monitoring peptides that may not contain tryptophan or tyrosine.

Materials and equipment

Protein solution to be measured

Table 1.1 Extinction coefficients of immunochemically relevant proteins. The values for $E^{1\%}_{280}$ (i.e. the absorbance of a 10 mg/ml solution at 280 nm) are averages of the heterogeneous proteins

	IgG	13.6
	IgM	11.8
	IgA	13.2
secretory	IgA	13.1
	IgD	17.0
	IgE	15.3
	γ chain	13.7
	μ chain	13.9
	α chain	15.5
	light chain	12.3
	J chain	6.8
secretory	component	12.7
	Fab	15.0
	Fc	12.0
	Fd	16.0
	V_H	27.0
	Concanavalin A	12.0
	Lens culinaris lectin	12.5
	Bovine serum albumin	6.7

Values for other proteins are listed in Fasman (1976).

Buffer in which the protein is dissolved
UV spectrophotometer
Quartz cells — 1 cm light path

Procedure

1 If necessary, centrifuge the sample to remove any particles or complexes in suspension.
2 Set the wavelength of the spectrophotometer to 280 nm and zero the absorbance with the buffer in one cell.
3 Read the absorbance of the sample either in the same cell or its matched twin. If the value obtained is greater than 2.0 dilute an aliquot of the sample (e.g. 1/5 or 1/10) or use shorter light path cells (e.g. 2 mm) until the reading falls between 0.1 and 1.5.
4 Repeat steps 2 and 3 at 260 nm.
5 Calculate the ratio of absorbance 260:280 nm. This should be below 0.6; high ratios indicate that the protein is contaminated with interfering substances, notably nucleic acids.

$$\text{concentration of sample} = \frac{\text{absorbance at 280 nm}}{\text{extinction coefficient at 280 nm}} \times 10 \text{ mg/ml}$$

6 For mixtures of proteins or for any protein with unknown extinction coefficient:

$$\text{protein concentration} = 1.55 \times \text{absorbance at 280 nm} - 0.77 \times \text{absorbance at 260 nm}$$

Remember to calculate the absorbance for the *original* solution in a 1 cm light path if it was diluted or measured in thinner cells.

1.1.2 **Folin phenol method** (modified from Lowry *et al.*, 1951)

This method is used widely when UV absorbance cannot be measured (see above). Several substances interfere with this assay and so a buffer blank should always be included. Non-ionic detergents form precipitates but the supernatant will usually give a valid result (see also note 3 and Section 1.1.3). Many modifications exist for circumventing these problems (Peterson, 1979).

Materials and equipment

Protein solution to be measured
Buffer in which the protein is dissolved
Any standard protein with known extinction coefficient (see step 1 below)
2% (w/v) copper sulphate, hydrated ($5H_2O$)
4% (w/v) sodium potassium tartrate
3% (w/v) sodium carbonate in 0.2 M sodium hydroxide
Folin and Ciocalteu's phenol reagent (BDH — Appendix 3)
Visible light spectrophotometer

Procedure

1 Make a 1 mg/ml solution of the standard protein and calculate the exact concentration from its absorbance at 280 nm (Section 1.1.1).
2 Pipette an aliquot of the unknown solution containing 5–50 μg of protein, the same volume of the buffer blank, and 0, 2, 5, 10, 20, 35, and 50 μl of the standard solution (the zero tube is the water blank) into separate tubes.
3 Add water to bring the contents of each tube to the same volume (ideally less than 200 μl but the assay will cope with up to 1 ml).
4 Mix 1 ml of copper sulphate solution and 1 ml of tartrate solution with 48 ml of carbonate solution (this mixture should be freshly prepared). Add 1 ml of this to each tube, mix and incubate for 10 min at room temperature.
5 Add 50 μl of phenol reagent to each tube, mix again and incubate for 25 min.
6 Mix again and 5 min later read the absorbance of each tube at 640 nm using the water blank to zero the spectrophotometer.
7 Plot the absorbance against protein content for the standard solution (this is not quite linear) and from this read off the amount of protein in the unknown. Subtract any absorbance of the buffer blank and calculate the protein concentration in the original solution from the volume taken for assay in step 2.

Notes

1 The standard protein should be structurally similar to that in the unknown solution if possible, because proteins vary somewhat in their colour yield. The standard solution can be aliquoted and stored frozen.

2 The lower limits of detection are about 2 μg in 200 μl and 5 μg in 1 ml; 50 μg in 200 μl has an absorbance of approximately 0.4.

3 Pierce (Appendix 3) have introduced an alternative reagent to Folin-Ciocalteu for detecting the Cu^+ produced by the protein — bicinchonic acid (BCA). This reagent is more stable and does not form precipitates with detergents (see above); the assay is simpler and is claimed to be more sensitive with a broader working range.

1.1.3 Dye-binding assays

Bradford (1976) introduced a protein assay based on the shift in absorbance maximum of Coomassie Brilliant Blue G, from 465 to 595 nm, when it binds to protein. It is simpler and less susceptible to interference by many substances than the Folin phenol method but it does not solubilize filamentous or membrane proteins as well.

The reagent is available commercially (Bio-Rad, Pierce — Appendix 3). Alternatively, dissolve 100 mg Coomassie Brilliant Blue G-250 in 50 ml 95% ethanol. Add 100 ml 85% (w/v) phosphoric acid, make up to 1 litre with water and filter through Whatman no. 1 paper (the reagent can be kept for a few weeks, re-filtering if a precipitate forms, but should be discarded after this time; the dye/ethanol stock keeps for years). Add 5 ml of the reagent to 100 μl of test solution (containing 10–100 μg protein), mix, leave for 5 min and read the absorbance at 595 nm. For a more sensitive assay make up the reagent to 200 ml instead of 1 litre and add 0.2–0.8 ml of test solution (containing 1–20 μg protein).

In common with most assays the colour yield varies somewhat between different proteins (see Section 1.1.2, note 1).

An alternative dye-binding assay has been developed recently (Winterbourne, 1986). This measures the amount of dye that binds to protein dried onto filter paper. It is more sensitive than the Bradford or Folin phenol, does not require protein to be in solution and is more specific for protein since small interfering molecules, e.g. peptides, are removed during the washing steps.

Materials and equipment

Protein solution or suspension to be measured
Standard protein (see Section 1.1.2, note 1)
0.4 g Coomassie Brilliant Blue R dissolved in 250 ml ethanol and 630 ml water
Glacial acetic acid