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IMMUNOCHEMICAL METHODS IN THE BIOLOGICAL SCIENCES: ENZYMES AND PROTEINS

R. J. Mayer
and J. H. Walker

Immunochemical Methods in the Biological Sciences: Enzymes and Proteins

R. J. Mayer
Senior Lecturer, Department of Biochemistry,
University of Nottingham Medical School,
Queen's Medical Centre, Clifton Boulevard,
Nottingham, NG7 2UH, UK

and

J. H. Walker
Research Fellow, Abteilung für Neurochemie,
Max Planck Institut für Biophysikalische Chemie,
Göttingen-Nikolausberg, Göttingen, GFR

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Preface

We would not have been stimulated to write this book without the excitement of using immunochemical techniques in the authors' laboratories. The excitement was often provided by the "unsung heroes" who helped to carry out many of the techniques. We therefore gratefully acknowledge the co-operation and skills of Ron, Linda, Janice, Janet, Elaine, Susan, Rowland, Ray, Reg, Alex, Pete, Trevor, Brian, Sue, Norman, Khalidah, Patrick and Jenny. We would like to thank Dr Colin Wild for indexing the book.

February, 1980

R. J. Mayer
J. H. Walker

To the late Professor G. H. A. Hübscher, Foundation Professor in Biochemistry, University of Nottingham Medical School, Nottingham, UK

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1
Introduction

I. General Introduction

The science of biochemistry can be distinguished, perhaps aphoristically, from chemistry by the way in which enzymes bring about what are otherwise thermodynamically unfavourable chemical reactions. Since it is also axiomatic that enzymes are proteins it is not surprising that many years of dedication have been devoted to the determination of protein structure and function: indeed, much of the last 20 years in biochemistry has been devoted to such studies and remarkable advances have been made, not least in the elucidation of the structures of the immunoglobulins themselves. Latterly more emphasis has been placed on studies of the ways in which enzyme activity can be regulated *in vitro* and particularly *in vivo*: here normal physiological control of enzyme activity has been resolved into rapid (acute) and slow (chronic) mechanisms. Acute regulation of enzyme activity is carried out without altering the amount of the particular regulatory enzyme(s), whereas chronic regulation is achieved by changing the amount(s) of key regulatory enzymes. Not only have the normal physiological regulators been defined (e.g. hormones), but also the effect of a large variety of exogenous agents has been studied. It can safely be said that, with a few very rare exceptions, the broad division into acute and chronic changes in enzyme activity and therefore physiological regulation of metabolism has been defined, and can only be further elaborated, by means of immunochemical techniques. The only satisfactory straightforward way to measure the amount of an enzyme or protein in a tissue extract is by means of these techniques. Chronic changes in enzyme or protein amounts in response to a variety of stimuli have, therefore, been shown predominantly by immunochemical methods.

Interestingly immunoisolation of enzymes or proteins from tissue extracts is the method of choice for many workers in the field of acute enzyme regulation. Here changes in enzyme activity are often mediated by protein modification (e.g. phosphorylation): antibodies can therefore be very effectively used to make the rapid isolation of modified species, which is needed in order to establish the extent of modification in different physiological or pathological states.

Studies of enzymes and proteins throughout the animal and plant kingdoms can be very effectively carried out immunochemically. It is salutory that interpretation of any work on physiological or pathological changes in enzyme activity in a tissue will ultimately hang on whether the amount or just the activity of an enzyme changes; this can only be effectively resolved immunochemically. Immunochemical reagents are being increasingly used for cell and molecular biological purposes. Enzymes and proteins in a variety of cell membranes and organelles are being probed immunochemically. These studies are leading to substantial advances in our understanding of membrane structure, function and turnover. Eventually biological scientists must ask if they can afford *not* to use immunochemical techniques.

Perhaps not surprisingly the greatest sympathy for these biological problems comes from biologists and not immunologists; a consequence of this is that the biologists must perfect the skills and techniques, albeit often established in principle by immunologists, to solve their specific problems in immunochemistry. The work described in this book is a personal account of an attempt made by two biochemists to grasp and solve many of the immunochemical problems which are routinely presented to biologists. As usual in science it is not that the techniques are not available but that the scientists studying particular scientific problems are not aware of them; alternatively designers of techniques do not know of all the uses to which their methods may be put. Scientific developments in a field are ultimately entwined in the scientific philosophy of their perpetrators. This is often overlooked and can be reduced to the desire to understand some particular scientific problem. Biochemistry is fundamental to all biological sciences and, therefore, this book is written for all biologists who may wish to start an immunochemical study of enzymes or proteins from scratch; it does not catalogue all the available techniques in immunology but tries to emphasize the developmental sequence of methods and techniques which can convert any biologist into a practising immunochemist.

II. Aims and Objectives

Immunochemistry is the study of the interaction between antigens (e.g.

proteins) and antibodies. Immunization of an animal produces antibodies against proteins which the animal recognizes to be "foreign". The immunoglobulins which are produced in response to this challenge are specific to the antigen (or antigens) injected. The immunoglobulin population which is specific to an antigen is heterogeneous, in that subpopulations of immunoglobulin exist which interact with different regions (or antigenic determinants) on the surface of the antigen.

A problem in writing a book on this topic is that there is a tendency to generalize about immunochemical processes which are by definition not general but very specific phenomena. The production, processing and use of each antiserum will vary depending on the antigen of interest. Furthermore biologists have very different interests in the properties of enzymes and proteins and, therefore, the requirements and objectives of each research group will be different. Naturally this will lead to the use of different methods for the identification, isolation and quantitation of antigens from tissue extracts: the purity of antibodies needed for a piece of work may vary from crude antiserum (e.g. for immunotitration of enzyme activity) to carefully purified antibodies (for immunoadsorbent chromatography or antigen quantitation). Potential immunochemists should consider their specific requirements carefully before choosing from the methods and procedures which are outlined in this book.

Immunochemical studies (with humoral antibodies, i.e. in the serum) have been carried out on enzyme activity, enzyme evolution, the nature of antigenic determinants on enzymes, immunogenicity in relation to enzyme structure and function, the relationship between proenzymes and enzymes and between apoenzymes and enzymes, multiple forms of enzymes and allostery. The results indicate the complex relationship between factors responsible for immunogenicity of a protein antigen, e.g. level of conformational organization, and the subsequent interaction of antibodies with the protein antigen (Arnon, 1973).

Sensitive immunochemical techniques are being used increasingly to measure the amount and rate of turnover (synthesis and degradation) of enzymes and proteins (Philippidis *et al.*, 1972; Speake *et al.*, 1976). Immunochemical methods have recently been developed to isolate polyribosomes containing immunoreactive nascent antigen chains, from which specific mRNA can be prepared (Palacios *et al.*, 1972).

Naturally there are difficulties in carrying out immunochemical studies on proteins and enzymes. Problems arise not only in connection with methodology but also in relation to the interpretation of experimental data, e.g. reactions of identity with unstable proteins or membrane proteins.

The purpose of this book is to review the methods involved in the

production, processing and use of antisera to proteins and enzymes. Each section is structured to emphasize the principles, practice, generality and problems of each technique. The book is not intended as a completely comprehensive guide to all immunochemical techniques which have been used to study proteins or enzymes. Such studies have been reviewed (Cinader, 1963, 1967; Clausen, 1971; Arnon, 1971, 1973; Kwapinski, 1972; Bjerrum and Bøg-Hansen, 1976b). The book is intended to be a practical guide to anyone wishing to raise and use antisera to proteins or enzymes. It is hoped that the contents will be useful to those involved in a study of soluble or membrane protein antigens in any biological system. The book is based in part on the authors' immunochemical studies of fatty-acid synthetase (Speake *et al.*, 1975, 1976), acetyl-CoA carboxylase (Manning *et al.*, 1976), 6-phosphogluconate dehydrogenase (Betts and Mayer, 1975, 1977), cytochrome oxidase (Walker and Mayer, 1976), monoamine oxidase (Dennick and Mayer, 1977), casein (Al-Sarraj *et al.*, 1978) and cholinergic vesicle proteins. The studies are related to the turnover of the named enzymes (Walker *et al.*, 1976) and the cholinergic mechanism of nervous transmission respectively.

2
Antiserum Production

Antisera may be produced against a single protein antigen (monospecific antiserum) or against a mixture of protein antigens (polyspecific or multispecific antiserum). For many immunochemical studies on enzymes or proteins it is advantageous to produce a monospecific antiserum (Walker *et al.*, 1976). However, polyspecific antisera may be produced deliberately to analyse complex protein mixtures (Bjerrum and Bøg-Hansen, 1976a). Alternatively, an antiserum may be raised to an antigen of interest prepared from a species which is not to be studied (e.g. beef heart cytochrome c oxidase has been used to raise an antiserum which was used to study the rat liver enzyme, Hackenbrock and Hammon, 1975; bovine dopamine β-hydroxylase has been used to raise an antiserum to study the rat brain enzyme, Ross *et al.*, 1978). Furthermore antisera may be prepared to commercially available antigens (e.g. Glutamine synthetase, Koch and Nielsen, 1975) although it is advisable to test the purity of the antigen before beginning the immunization schedule. Such an assessment may indicate that further purification of an antigen is necessary before immunization can be started.

I. Preparation of Antigens

A. Antigen Purity

As a working principle it should always be assumed that an antigen which is pure by biochemical criteria (e.g. single component in protein analytical systems) is not pure immunologically. This assumption can avoid tremendous disappointment after a lengthy immunization schedule. There are many examples of biochemically pure antigens giving rise to polyspeci-

fic antisera (Clausen, 1971) and in our own laboratory only one protein antigen (fatty acid synthetase) out of many has given rise to a monospecific antiserum.

The major dilemma for the enzymologist is to decide how far to purify the antigen, measured in terms of time and effort, given that there are a variety of procedures for antiserum purification (Chapter 3), and that however hard he tries his antigen may still be impure. As usual, experience is the only guide, but in general it is best to obtain the most purified preparation for use as antigen, knowing that the antiserum can subsequently be purified if necessary.

B. Nature of Antigen Preparation

Enzyme and protein purification procedures give rise to preparations in buffer solutions of different pH and ionic strength which can contain a variety of agents which have been included to protect the protein from inactivation (e.g. dithiothreitol, 2-mercaptoethanol, EDTA, glycerol, stabilizing ions and coenzymes). Work with membrane protein antigens usually means the isolation and purification of a protein in the presence of detergents (often non-ionic) or at very high salt concentrations (e.g. 3 M KCl for plasma membrane antigens, Price and Baldwin, 1977). These agents may affect the microheterogeneity of antibody subpopulations (e.g. by masking antigenic determinants), but in general there is little evidence that they affect antibody production. Antisera to membrane antigens in detergents (e.g. Triton X-100) are not difficult to produce (e.g. Dennick and Mayer, 1977).

Although ionic detergents (e.g. sodium dodecyl sulphate) are known to disrupt antigen–antibody interactions (Crumpton and Parkhouse, 1972; Bjerrum and Bøg-Hansen, 1976b) antisera have been successfully raised to protein subunits isolated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate. For example, Strauss *et al.* (1975) homogenized antigen-containing gel in phosphate-buffered saline and injected it into rabbit foot pads. Similarly immunization with single bands obtained by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate has been successfully used to raise monospecific antisera to actin (Blomberg *et al.*, 1977), microtubule associated protein (Sherline and Schiavone, 1977) and myosin (Tashiro and Stadtler, personal communication). It must be remembered that polypeptides of similar molecular weight may be present in a single piece (band) of polyacrylamide gel, e.g. tubulin and a polypeptide associated with neurofilaments (Gilbert,1978). However, immunization with antigens prepared in this way has great potential for membrane antigens which are often very difficult to

resolve, purify and identify in systems which do not contain sodium dodecyl sulphate. Protein subunits prepared in sodium dodecyl sulphate will more frequently be used as antigens, as more laboratories use two dimensional polyacrylamide gel electrophoresis to purify membrane protein subunits (Dale and Latner, 1969; O'Farrell, 1975). It is often easier to prepare a protein subunit reproducibly by these methods than to try and purify native proteins from some cellular organelle (e.g. synaptic vesicle membrane proteins).

It is tempting to suggest that all so-called purified proteins should be subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate; following staining the subunit or subunits of the antigen of interest could be cut out, pooled and used for the preparation of samples for immunization. This approach could minimize the production of antibodies to contaminating antigens, except, of course, to those contaminants which have the same subunit size(s) as the antigen of interest. Excellent antiserum to human liver monoamine oxidase has recently been produced in this way (Russell *et al.*, 1978a).

However, the problems of interpretation associated with the use of antisera to subunits of functional protein complexes in membranes must be recognized. For example antisera raised to subunits isolated from multisubunit enzyme complexes may not cross-react with the holoenzyme (Werner, 1974).

C. Antigen Mixtures

Many polyspecific antisera have been prepared to protein mixtures and have been used to identify and quantify soluble (e.g. Weeke, 1973b) and membrane (e.g. Bjerrum and Bøg-Hansen, 1976a) antigens. Polyspecific antisera can be used to study qualitative and quantitative changes in antigen populations (e.g. in membrane fusion or during chronic enzyme adaptation in tissues).

If the resolution of crossed-rocket immunoelectrophoresis is sufficiently good, immunoprecipitates can be cut from the agarose gel and used to produce monospecific antisera (Koch and Nielsen, 1975). However, it is very useful to be able to identify the biochemical nature of the antigen in a particular immunoprecipitate if the subsequent monospecific antiserum is to be of maximal use in biological experiments. Identification of an enzyme which retains activity in an immunoprecipitate is relatively simple (Blomberg and Perlman, 1971a; Clausen, 1971). Similarly, proteins which bind calcium (Suttie *et al.*, 1977), epinephrine (Blomberg and Berzins, 1975), or which are phosphorylated (Gordon *et al.*, 1977) are easy to identify in immunoprecipitates after the incorporation of radio-labelled ligand or

through change in the electrophoretic mobility of the antigens after binding of the ligand. Identification of enzymically inactive antigens in immunoprecipitates can be difficult and equivocal and, paradoxically, often requires the use of purified antigen to identify the rocket immunoprecipitate of interest. However, the increasing number of methods which are becoming available to characterize antigens in immunoprecipitates (Chapter 4) will make this problem much simpler.

The relative immunogenicities of proteins in a mixture are of great importance when considering the outcome of an immunization schedule designed to obtain a polyspecific antiserum. The problem is that some proteins may be much better macromolecular antigens than others, thus complicating the subsequent use of the antiserum (e.g. in immunoprecipitation analyses in gels).

Indeed, in some cases it may not be possible to obtain precipitating antibodies to some of the major protein species in an antigen mixture, while minor protein species may give rise to high titres of antibodies (e.g. in cholinergic synaptic vesicles). It may be that a single protein in a protein mixture may be the only effective antigen in the mixture. Injection of whole chromaffin granules seems to give rise to a monospecific antiserum to dopamine β-hydroxylase (Helle *et al.*, 1979).

When preparing polyspecific antisera to membrane antigens, contaminating cytosolic proteins may act as excellent antigens giving much better antibody production than the membrane antigens. Furthermore, even when the intention is to produce a monospecific antiserum (e.g. to purified integral mitochondrial membrane enzymes) contaminants in the preparations can give much larger titres of antibodies than the antigens of interest (e.g. Walker *et al.*, 1976; Dennick and Mayer, unpublished data).

II. Preparation of Antigens for Immunization

A. Adjuvants

Protein antigens are usually mixed with material that will increase the concentration of circulating antibodies, i.e. adjuvants. There are a variety of adjuvants which may themselves be antigenic (e.g. tubercle bacilli) or not (mineral oil) but which can improve the humoral immune response by mechanisms which include increasing the number of cells involved in antibody formation, assuring a more efficient processing of antigens and prolonging the duration of the antigen in the immunized animal. The most commonly used adjuvant is complete Freund's adjuvant, which consists of killed mycobacteria (e.g. tubercle bacilli), an oil and an emulsifier. In